Bacteria colonizing paper machines

Jaakko Ekman

Department of Food and Environmental Sciences
Faculty of Agriculture and Forestry
University of Helsinki

Academic dissertation in Microbiology

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Supervisor: Prof. Mirja Salkinoja-Salonen
Department of Food and Environmental Sciences
Faculty of Agriculture and Forestry
University of Helsinki
Helsinki, Finland

Reviewers: Prof. Dr. Dr.-Ing. Peter Kämpfer
Institute of Applied Microbiology
Faculty of Agricultural Sciences, Nutritional Sciences and Environmental Management
Justus-Liebig-University Gießen
Gießen, Germany

Doc. Dr. Anna Maria Pirttilä
Department of Biology
Faculty of Science
University of Oulu
Oulu, Finland

Oppnent: Prof. Dr. Tjakko Abee
Laboratory of Food Microbiology
Wageningen University
Wageningen, The Netherlands

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Front cover: Images in clockwise order from upper left corner: **1.** Orange biofilm on the surfaces of an empty wire pit from a paper machine. **2.** Green fluorescent protein labelled cells of *Bacillus thuringiensis* strain BT-1. **3.** Field emission scanning electron micrograph of biofilm pellicle of *Bacillus cereus* strain F4810/72 (courtesy of Mari Raulio). **4.** Biofilm samples collected from a paper machine. Sample number 20 was collected from the wire pit in image 1.

Ansalle ja Allille

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List of Original Publications

I. Ekman J, M Kosonen, S Jokela, M Kolari, P Korhonen and M Salkinoja-Salonen (2007). Detection and quantitation of colored deposit-forming *Meiothermus* spp. in paper industry processes and end products. *Journal of Industrial Microbiology & Biotechnology*. 34:203-211.

II. Ekman J, I Tsitko, A Weber, C Nielsen-LeRoux, D Lereclus, M Salkinoja-Salonen (2009). Transfer of *Bacillus cereus* spores from packaging paper into food. *Journal of Food Protection*. 72:2236-2242.

III. Ekman J, M Raulio, H-J Busse, D Fewer, M Salkinoja-Salonen (2011). *Deinobacterium chartae* gen. nov., sp. nov., an extremely radiation resistant biofilm forming bacterium isolated from a Finnish paper mill. *International Journal of Systematic and Evolutionary Microbiology*. 61:540-548.

IV. Ekman J, A Kruglov, M A Andersson, R Mikkola, M Raulio, M Salkinoja-Salonen. Why do certain *Bacillus cereus* strains produce cereulide? Submitted.

The Author's Contribution

I. Jaakko Ekman carried out most of the qPCR experiments and isolated majority of the novel bacterial strains. He interpreted the results and wrote the paper together with the other authors.

II. Jaakko Ekman planned and carried out the experimental work excluding construction of the GFP-labelled strain and contact agar experiments. He interpreted the results, wrote the paper together with the other authors and was the corresponding author.

III. Jaakko Ekman planned and executed major part of the experimental work. He interpreted the results, wrote the article together with the other authors and was the corresponding author.

IV. Jaakko Ekman planned and carried out the experimental work excluding the K^+ leakage measurements and quantification of cereulide. He interpreted the results, wrote the paper together with the other authors and was the corresponding author.

Abbreviations

AI-2	Autoinducer 2
ATCC	American Type Culture Collection
ATP	Adenosine 5'-triphosphate
APL	Aminophospholipid
Gen. nov.	Genus novum
BHI	Brain heart infusion
BCM	*Bacillus cereus* group plating medium
CFU	Colony forming unit
CMC	Carboxymethyl cellulose
DPD	4,5-dihydroxy-2,3-pentadiene
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH
eDNA	Extracellular DNA
EPS	Extracellular polymeric substances
FESEM	Field emission scanning electron microscope
JC-1	5,5',6,6'-tetrachloro-1,1',3,3' tetraethylbenzimidazolylcarbocyanine iodide
K_{ow}	Octanol-water partition coefficient
LB	Luria-Bertani
MYP	Mannitol-egg yolk-polymyxin
NA	Nutrient agar
NRPS	Non-ribosomal peptide synthetase
PCA	Plate count agar
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
qPCR	Quantitative PCR
PVC	Polyvinyl chloride
rRNA	Ribosomal RNA
Sp. nov.	Species novum
Spp.	Species (plural)
SMM	Skim milk medium
TSA	Tryptic soy agar
TSB	Tryptic soy broth

Glossary

ABC transporter	transmembrane proteins utilizing energy of ATP hydrolysis to transport substrates across membranes
activated methyl cycle	metabolic cycle generating S-adenosyl-L-methionine providing activated methyl groups for the methylation of *e.g.* proteins, DNA and RNA.
autoinducer	a signaling molecule in quorum sensing
biofilm	multicellular communities held together by a self-produced extracellular matrix
broke	repulped paper from mill's own production
chaotrophic	solutes disrupting hydrogen bonding between water molecules
depsipeptide	a peptide in which some of the peptide bonds are replaced by ester bonds
electrochemical gradient	diffusion gradient of charged molecules that takes into account both electrical potential and chemical concentration difference across the membrane
emetic	causing emesis, *i.e.* makes you vomit
extrusion layer	coating of paper from plastic material
fruiting body	multicellular, upwards growing structure where sporulation takes place
headbox	part of paper machine; spreads the fiber suspension onto the wire
kosmotrophic	solutes stabilizing hydrogen bonding between water molecules
nonribosomal peptide synthetase	enzymatic machinery producing peptides without ribosomes
oligotrophic	having low level of nutrients
paracrine signaling	a type of signaling where signal molecule is released by other cells than those responding to it.
pellicle	biofilm growing at the air-liquid interphase, *i.e.* not attached to a solid surface
peptide	a short (2 - ~50) polymer of amino acids
pleiotrophic regulator	a regulator controlling the expression of several genes
polyphasic taxonomy	takes into account all available data (both phenotypic and genotypic)
press felt	woven fabric transporting paper sheet into the press section
press section	part of paper machine where water is removed from the paper by mechanical pressing

quorum sensing	population density dependent signaling of bacteria where same cells produce the signal and respond to it
sensu lato	in the broad sense
sensu stricto	in the narrow sense
wet-end	paper machine area where paper is being formed from its ingredients
wire	fabric on which the pulp slurry forms a sheet of paper when water drains away through it
white water	general term for paper machine waters which have cloudy appearance due to dispersed fibers

Abstract

Bacteria growing in paper machines can cause several problems. Biofilms detaching from paper machine surfaces may lead to holes and spots in the end product or even break the paper web leading to expensive delays in production. Heat stable endospores will remain viable through the drying section of paper machine, increasing the microbial contamination of paper and board. Of the bacterial species regularly found in the end products, *Bacillus cereus* is the only one classified as a pathogen. Certain *B. cereus* strains produce cereulide, the toxin that causes vomiting disease in food poisonings connected to *B. cereus*.

The first aim of this thesis was to identify harmful bacterial species colonizing paper machines and to assess the role of bacteria in the formation of end product defects. We developed quantitative PCR methods for detecting *Meiothermus* spp. and *Pseudoxanthomonas taiwanensis*. Using these methods I showed that *Meiothermus* spp. and *Psx. taiwanensis* are major biofoulers in paper machines. I was the first to be able to show the connection between end product defects and biofilms in the wet-end of paper machines. I isolated 48 strains of primary-biofilm forming bacteria from paper machines. Based on one of them, strain K4.1^T, I described a novel bacterial genus *Deinobacterium* with *Deinobacterium chartae* as the type species.

I measured the transfer of *Bacillus cereus* spores from packaging paper into food. To do this, we constructed a green fluorescent protein (GFP) labelled derivative of *Bacillus thuringiensis* and prepared paper containing spores of this strain. Chocolate and rice were the recipient foods when transfer of the labelled spores from the packaging paper to food was examined. I showed that only minority of the *Bacillus cereus* spores transferred into food from packaging paper and that this amount is very low compared to the amount of *B. cereus* naturally occurring in foods. Thus the microbiological risk caused by packaging papers is very low.

Until now, the biological function of cereulide for the producer cell has remained unknown. I showed that *B. cereus* can use cereulide to take up K^+ from environment where K^+ is scarce: cereulide binds K^+ ions outside the cell with high affinity and transports these ions across cell membrane into the cytoplasm. Externally added cereulide increased the growth rate of cereulide producing strains in medium where potassium was growth limiting. In addition, cereulide producing strains outcompeted cereulide non-producing *B. cereus* in potassium deficient environment, but not when the potassium concentration was high. I also showed that cereulide enhances biofilm formation of *B. cereus.*

Tiivistelmä

Paperitehtailla kasvavat bakteerit aiheuttavat monia ongelmia. Paperikoneen teräspinnoilta irtoavat biofilmipalat voivat näkyä reikinä ja läikkinä lopputuotteessa, tai jopa katkaista paperirainan johtaen kalliiseen tuotannon keskeytykseen. Kuumuutta kestävät bakteeri-itiöt säilyvät elävinä paperikoneen kuivausosan läpi heikentäen paperin tai kartongin hygieenistä laatua. Paperista yleisesti löydettävistä bakteerilajeista *Bacillus cereus* on ainoa, joka luokitellaan ihmiselle patogeeniseksi. Jotkin *B. cereus* –kannat tuottavat kereulidia, toksiinia joka voi aiheuttaa ihmisille jopa kuolemaan johtavan ruokamyrkytyksen.

Väitöskirjatyöni ensimmäinen tavoite oli tunnistaa paperikoneilla kasvavia haitallisia bakteerilajeja ja selvittää niiden osuus paperin likaläikkien syntyyn. Kehitimme kvantitatiiviset PCR menetelmät *Meiothermus*-bakteerisuvun ja *Pseudoxanthomonas taiwanensis* –bakteerilajin tutkimiseen. Näillä menetelmillä osoitin, että *Meiothermus*- ja *Pseudoxantomonas* -bakteerit ovat yleisiä paperitehtaiden biofilmeissä ja kiertovesissä. Osoitin myös yhteyden paperikoneen märkäpään biofilmien ja lopputuotteiden likäläikkien välillä: molemmista löytyi suuria määriä samojen bakteerilajien DNA:ta. Väitöskirjatyöni aikana eristin paperitehtailta 48 sellaista bakteerikantaa, jotka pystyvät tarttumaan puhtaalle pinnalle ja kasvamaan biofilminä paperitehdasolosuhteissa. Yksi niistä, kanta K4.1^T, edustaa tieteelle aiemmin tuntematonta bakteerisukua. Tämän kannan perusteella kuvasin uuden bakteerisuvun ja –lajin, *Deinobacterium chartae*.

Työni toinen tavoite oli selvittää elitarvikepakkauskartongista ruokaan siirtyvien *Bacillus cereus* –itiöiden määrä. Käytin tähän kehittämäämme fluoresoivalla proteiinilla (GFP) leimattua bakteerikantaa. Valmistimme kartonkia, joka sisälsi leimattuja bakteeri-itiöitä ja tutkimme kuinka suuri osa itiöistä siirtyy kartongista suklaaseen ja riisiin. Osoitin, että ruokaan siirtyneiden itiöiden osuus oli hyvin pieni, vain 0,01 –0,3% kartongin sisältämistä *B. cereus* –itiöistä. Ruuissa on luontaisesti *B. cereus* –bakteereja, ja niiden määrä on usein paljon suurempi kuin kokeessamme ruokaan siirtyneiden itiöiden määrä. Täten pakkauskartonkien aiheuttama mikrobiologinen riski ruuan turvallisuudelle on pieni.

Selvitin myös mitä hyötyä kereulidia tuottava bakteeri saa itselleen tästä ihmiselle erittäin myrkyllisestä molekyylistä. Osoitin, että *B. cereus* pystyy hyödyntämään ympäristön kaliumvarantoja paremmin kereulidin avulla. Lisätty kereulidi nopeutti kereulidia tuottavien *B. cereus* -kantojen kasvua ympäristössä, jossa kaliumia oli niukasti tarjolla. Tässä ympäristössä nämä bakteerit pystyivät myös tehokkaasti kilpailemaan sellaista *B. cereus* -

kantaa vastaan, joka ei tuota kereulidia. Lisäksi osoitin, että kereulidi lisää *Bacillus cereus* -kantojen kasvua biofilmeinä.

1. Review of the Literature

1.1 Paper machine as a habitat for bacteria

The process water in paper machines provides growth conditions suitable for bacteria. Water temperature is 40 - 60 °C and pH 5-10. Process waters are rich in organic carbon (cellulose, starch), but other nutrients, such as nitrogen, may be growth limiting (Väisänen *et al.* 1998, Kolari *et al.* 2003, Kolari 2007, Kanto Öqvist *et al.* 2008). Process changes, such as reduced water consumption, faster running machines, increased use of coatings and fillers, and transition to neutral or alkaline processes, have increased problems caused by bacteria (Blanco *et al.* 1996).

1.2 Problems in paper machines caused by bacterial growth

Biofilms detaching from paper machine surfaces may cause holes and coloured spots in paper, reducing the quality of the end product. Detaching biofilms can also break the paper web leading to downtime and production losses (Blanco *et al.* 2004, Kolari 2007). Haapala *et al*. (2010) noted that over 60 % of the web breaks were caused by holes or deposits on paper web. Most of the deposits and edges of the holes contained bacterial DNA indicating microbial involvement in the formation of the deposit (Haapala *et al*. 2010).

Anaerobic micro-organisms in paper mills can produce smelly compounds, such as hydrogen sulphide (H_2S) and volatile fatty acids, that lead into complains from the neighbouring community or smell in the end products (Blanco *et al.* 2004, Kanto Öqvist *et al.* 2008). This is a problem especially on machines using recycled fibres and/or with a closed water cycle. H_2S, produced by sulphate reducing bacteria or archaea, can also cause even fatal intoxications in poorly ventilated areas (Kolari 2007).

Heat stable endospores will remain viable through the drying section of paper machines. Therefore spore forming bacteria in the paper machine wet-end can increase the microbial contamination of paper mill end products. This needs attention especially with machines producing food quality papers (Väisänen *et al.* 1991, Suihko & Stackebrandt 2003, Priha *et al.* 2004).

Some bacteria, for example amylolytic *Bacillus* species, can spoil surface sizing materials of paper making (Väisänen *et al.* 1989, Väisänen *et al.* 1998). The action of many different bacterial groups, for example sulphate reducers and producers of thiosulfate or of oxalate,

may lead to microbially induced corrosion of steel surfaces (Soimajärvi *et al.* 1978, Uutela *et al.* 2003, Blanco *et al.* 2004).

1.3 Bacteria found in paper machines and in their end products

Bacteria enter the paper mill mostly in water and raw materials. Large amounts of bacteria enter the machine especially when recycled fibres are used (Blanco *et al.* 1996). Väisänen *et al*. (1998) isolated 390 bacterial strains from a single paper machine, demonstrating the huge bacterial diversity present at paper machines. It has been proposed that each machine has its own, unique bacterial population adapted to that particular environment (Kolari 2007). Those bacterial species that have been reported in the litrature from paper machines in multiple studies are listed in Table 1.

Table 1. Bacteria found from paper machines. Both isolates and findings with DNA-based methods are included.

Bacterial genus	Species	Isolation site	References
Achromo-bacter	*A.piechaudii, A. xylosoxidans*	Warm water, headbox, spray water	Väisänen *et al.* 1998, Lindberg *et al.* 2001b
Acidovorax	*A. delafieldii*	Slime from wire section	Väisänen *et al.* 1998, Desjardins & Beaulieu 2003, Kashama *et al.* 2009
Acinetobacter	*A. lwoffii, A. radioresistens, A. baumannii*	Slime, pulp	Väisänen *et al.* 1998, Rättö *et al.* 2001, Suihko & Skyttä 2009
Arthrobacter	*A. agilis, A. ilicis*	End product, pulp	Väisänen *et al.* 1998, Oppong *et al.* 2000, Suihko & Skyttä 2009
Bacillus	*B. amyloliquefaciens, B. atrophaeus, B. cereus, B. circulans, B. coagulans, B. firmus, B. fusiformis, B. halodurans, B. jeotgali, B. licheniformis, B. megaterium, B. mycoides, B. pumilus, B. simplex, B. smithii, B. sphaericus, B. subtilis, B. thuringiensis*	Throughout the process, including end products	Väisänen *et al.* 1989, Väisänen *et al.* 1994, Pirttijärvi *et al.* 1996, Suominen *et al.* 1997, Väisänen *et al.* 1998, Pirttijärvi 2000, Pirttijärvi *et al.* 2001, Lindberg *et al.* 2001a, Lindberg *et al.* 2001b, Kolari *et al.* 2001, Raaska *et al.* 2002, Desjardins & Beaulieu 2003, Priha *et al.* 2004, Suihko *et al.* 2004, Kashama *et al.* 2009
Brevibacillus	*B. agri, B. brevis, B. laterosporus*	Slime from wire section, end product	Väisänen *et al.* 1989, Väisänen *et al.* 1991, Pirttijärvi *et al.* 1996, Suominen *et al.* 1997, Väisänen *et al.* 1998, Pirttijärvi 2000, Desjardins & Beaulieu 2003

Brevundimonas	*B. vesicularis, B. diminuta*	Slime, pulp	Verhoef *et al.* 2002, Suihko & Skyttä 2009
Burkholderia	*B. caryophylli, B. cepacia, B. kururiensis, B. multivorans*	Slime in wire section, white water, headbox, broke, CMC-slurry, machine chest, ground wood, pulp, end product	Väisänen *et al.* 1994, Väisänen *et al.* 1998, Lindberg *et al.* 2001a,Lindberg *et al.* 2001b, Kolari *et al.* 2003, Suihko & Skyttä 2009, Rasimus *et al.* 2010
Chelatococcus		Headbox, pulp	Suihko & Skyttä 2009, Prince *et al.* 2009
Citrobacter	*C. freundii*	Pulp, starch-based glue	Raaska *et al.* 2002, Priha *et al.* 2004, Suihko & Skyttä 2009
Cloaci-bacterium	*C. normanense*	Steel surfaces, pulp	Suihko & Skyttä 2009, Tiirola *et al.* 2009
Clostridium	*C. intestinale, C. magnum*	Broke, starch, pulp, slime, end product, sizing agent	Suihko *et al.* 2005
Deinococcus	*D. geothermalis, D. grandis*	Biofilms throughout the paper machine.	Väisänen *et al.* 1998, Oppong *et al.* 2000, Kolari *et al.* 2001, Peltola *et al.* 2008
Enterobacter	*E. amnigenus, E. cloacae, E. hormaechei, E. kobei, E. radicincitans, E. sakazakii*	Slime from wire section, pulp, starch-based glue, end product	Raaska *et al.* 2002, Desjardins & Beaulieu 2003, Rättö *et al.* 2006, Suihko & Skyttä 2009
Enterococcus	*E. durans, E. casseliflavus*	Slime in wire section, Pulp	Väisänen *et al.* 1998, Suihko & Skyttä 2009
Exiguo-bacterium	*E. acetylicum, E. aestuarii, E. marinum, E. mexicanum*	Pulp, slime from wire section	Väisänen *et al.* 1998, Kashama *et al.* 2009, Suihko & Skyttä 2009
Geobacillus	*G. kaustophilus, G. stearothermophilus*	Headbox, end product	Pirttijärvi 2000, Suihko *et al.* 2004, Prince *et al.* 2009
Hydrogeno-phaga	*H. palleronii*	Slime from wire section, pulp, bentonite slurry	Väisänen *et al.* 1998, Desjardins & Beaulieu 2003, Kashama *et al.* 2009
Klebsiella	*K. pneumoniae, K. oxytoca, K. variicola*	Wet-end surfaces, CMC slurry, white water, headbox, broke, spray water, pulp	Väisänen *et al.* 1994, Väisänen *et al.* 1998, Rättö *et al.* 2006, Suihko & Skyttä 2009

Meiothermus	*M. silvanus*	Headbox, slime from wire section	Kolari *et al.* 2003, Prince *et al.* 2009
Methylo-bacterium	*M. mesophilicum, M. zatmanii, M. extorquens*	Kaolin, white water flume, pulp, slime	Väisänen *et al.* 1998, Oppong *et al.* 2000, Verhoef *et al.* 2003, Suihko & Skyttä 2009
Micro-bacterium	*M. esteraromaticum, M. testaceum, M. maritypicum, M. paraoxydans, M. phyllosphaerae*	Slime from wire section, kaolin, pulp	Väisänen *et al.* 1998, Desjardins & Beaulieu, 2003, Suihko & Skyttä 2009
Micrococcus	*M. luteus*	Formulated mineral pigment, pulp	Väisänen *et al.* 1998, Suihko & Skyttä 2009
Nocardiopsis	*N. alba, N. dassonvillei, N. umidischolae, N. composta, N. prasina*	Wet-end surfaces, pulp, water	Oppong *et al.* 2003, Suihko *et al.* 2006
Paenibacillus	*P. amylolyticus, P. macerans, P. pabuli, P. polymyxa, P. stellifer, P. validus*	Broke, bentonite, end product, starch-based glue	Väisänen *et al.* 1991, Pirttijärvi *et al.* 1996, Väisänen *et al.* 1998, Raaska *et al.* 2002, Suominen *et al.* 2003, Suihko *et al.* 2004
Pantoea	*P. agglomerans*	Starch slurry, slime in wire section	Väisänen *et al.* 1994, Väisänen *et al.* 1998
Pseudomonas	*P. aeruginosa, P. fluorescens, P. putida, P. stutzeri, P. monteilii, P. plecoglossicida*	Headbox, slime form wire section, pulp, kaolin, starch-based glue	Väisänen *et al.* 1994, Väisänen *et al.* 1998, Rättö *et al.* 2001, Raaska *et al.* 2002, Desjardins & Beaulieu 2003, Kolari *et al.* 2003, Rochex *et al.* 2004, Kashama *et al.* 2009, Suihko & Skyttä 2009, Prince *et al.* 2009
Pseudo-xanthomonas	*P. taiwanensis*	Headbox, slime form wire section, pulp, broke, water	Desjardins & Beaulieu 2003, Suihko *et al.* 2004, Kashama *et al.* 2009, Prince *et al.* 2009, Suihko & Skyttä 2009
Ralstonia	*R. pickettii, R. solanacearum, R. mannitolilytica*	Hot water, broke, shower and warm water, headbox, bentonite, pulp	Väisänen *et al.* 1998, Lindberg *et al.* 2001a, Lindberg *et al.* 2001b, Suihko & Skyttä 2009,
Rhizobium		Slime from wire section, pulp	Desjardins & Beaulieu, 2003, Suihko & Skyttä, 2009
Schlegelella		Headbox, pulp	Suihko & Skyttä 2009, Prince *et al.* 2009
Sphaerotilus	*S. natans*	Slime from wire section	Pellegrin *et al.* 1999, Rättö *et al.* 2001

Sphingomonas	*S. capsulata, S. paucimobilis,* subclades *S. trueperi* and *S. aquatilis*	Slime from wire and press section, spray, warm and wire water	Väisänen *et al.* 1994, Väisänen *et al.* 1998, Desjardins & Beaulieu 2003, Rasimus *et al.* 2010, Kurissery *et al.* 2010
Staphylo-coccus	*S. epidermidis, S. haemolyticus, S. warneri*	Slime from wire section, pulp, bentonite slurry, Gypsum slurry	Väisänen *et al.* 1998, Desjardins & Beaulieu 2003, Kashama *et al.* 2009, Suihko & Skyttä 2009
Streptomyces	*S. albidoflavus, S. thermocarboxydus, S. cavourensis, S. diastaticus*	Wet-end surfaces, pulp, water	Oppong *et al.* 2003, Suihko *et al.* 2006
Steno-trophomonas	*S. rhizophila, S. maltophilia*	Pulp	Kashama *et al.* 2009, Suihko & Skyttä 2009
Tepidimonas		Headbox, pulp	Suihko & Skyttä 2009, Prince *et al.* 2009, Tiirola *et al.* 2009
Xanthobacter	*X. agilis*	Slime from wire section	Väisänen *et al.* 1998, Desjardins & Beaulieu 2003

Following species have been reported once from paper machines: *Amycolatopsis fastidiosa* (Suihko *et al.* 2004), *Bordetella avium* and *Cellulomonas flavigena* (Väisänen *et al.* 1998), *Cellulosimicrobium funkei, Leclercia adecarboxylata, Listeria innocua, L. monocytogenes, Ochrobactrum pseudintermedium, Oerskovia enterophila, Roseomonas cervicalis, Serratia rubidaea* and *Skermanella aerolata* (Suihko & Skyttä 2009), *Chryseobacterium indologenes* (Rättö *et al.* 2001), *Clavibacter michiganensis* (Väisänen *et al.* 1994), *Desulfovibrio desulfuricans, D. longreachensis, D. oxamicus, D. termitidis* (Maukonen *et al.* 2006) *Escherichia coli* (Kanto Öqvist *et al.* 2008), *Flectobacillus major* (Oppong *et al.* 2003), *Rahnella aquatilis, Raoultella planticola, R. terrigena* (Rättö *et al.* 2006), *Rubellimicrobium thermophilum* (Denner *et al.* 2006), *Sporosarcina globispora*(Väisänen *et al.* 1991), *Thermoanaerobacter thermohydrosulfuricus, Thermoanaerobacterium thermosaccharolyticum* (Suihko *et al.* 2005) and *Thermomonas haemolytica* (Busse *et al.* 2002). In addition, representatives of the following genera have been reported: *Aeromonas, Allorhizobium, Azorhizophilus, Azospirillum, Blastobacter* and *Leptothrix* (Desjardins & Beaulieu 2003), *Asticcacaulis* (Kashama *et al.* 2009), *Corynebacterium, Novosphingobium, Pannonibacter, Cupriavidus* (Suihko & Skyttä 2009), *Hydrogenophilus* (Prince *et al.* 2009), *Rhodobacter* (Tiirola *et al.* 2009), *Desulfotomaculum* (Mattila, 2002) and *Methanothrix* (Kanto Öqvist *et al.* 2008).

1.3.1 The bacterial contaminants of food packaging papers

Viable bacteria present in food packaging papers consist mainly of bacteria forming heat stable endospores (Väisänen *et al.* 1991, Pirttijärvi *et al.* 1996, Johansson *et al.* 2001, Suihko & Stackebrandt 2003) because the vegetative cells are more easily heat killed in the drying section, where surface temperatures reach up to 140ºC (Väisänen *et al.* 1998). Species found from paper industry end products are listed in Table 1. Densities of bacterial spores in food packaging papers vary from less than 50 up to 10^5 viable spores/g of paper. *Bacillus* and *Paenibacillus* have been reported as the most prevalent genera (Väisänen *et al.* 1991, Pirttijärvi *et al.* 1996, Suominen *et al.* 1997, Suihko & Stackebrandt 2003). *Bacillus cereus*

is the only species classified as a pathogen (Anonymous 2010) among the bacteria regularly found in the end products. The studies of Väisänen *et al.* 1991, Pirttijärvi *et al.* 1996 and Suihko & Stackebrandt 2003 showed that five to ten % of the viable spores in the end products represented *Bacillus cereus sensu lato,* which means the genetically highly conserved "*B. cereus* group", consisting of *B. cereus, B. thuringiensis, B. weihenstephanensis, B. mycoides, B. pseudomycoides* and *B. anthracis* (Stenfors Arnesen *et al.* 2008). Priha *et al*. (2004) used quantitative PCR for detecting DNA of the *B. cereus* group bacteria from paper industry end products with primers described earlier (Hansen & Hendriksen 2001, Hansen *et al.* 2001). They found *B. cereus* group from 3 out of nine samples, in amounts corresponding to $10^2 – 10^3$ cfu/g of paper (Priha *et al.* 2004).

Suominen *et al*. (1997) studied the distribution and growth of bacteria in food packaging paperboards. They used mainly paperboards extruded with polyethene and found that most bacteria were located in the interphase between the polyethene extrusion layer and the web of cellulose fibres. Bacteria enclosed inside the fibre web did not multiply, not even during 90 d exposure to food and moisture. Bacteria in the interphase of the fibreweb and the polyethene layer did multiply, but did not migrate to food under laboratory conditions simulating packaged food. The authors concluded that the main package-related threat to food hygiene were microbially contaminated starches (used as surface sizers) and mineral pigment coatings (Suominen *et al.* 1997). Johansson *et al*. (2001) studied the transfer of bacteria from paper to blood agar. They found that during 20 h contact time (at 4°C) less than 0.1 % of the total bacteria in the paper transferred to the agar surface (Johansson *et al.* 2001).

1.3.2 *Bacillus cereus* in paper machines

B. cereus has been isolated from slimes in the wire section (Väisänen *et al.* 1989, Väisänen *et al.* 1998), white water and calendar water (Pirttijärvi *et al.* 1999) and steel coupons immersed in water circuits (Kolari *et al.* 2001). Suihko *et al*. (2004) found *B. cereus* from all four mills investigated. They isolated 27 strains of *B. cereus* from broke, slime, water, pulp and chemical samples and from end products. Thus, *B. cereus* is a common contaminant in paper machines and can be isolated from many different sites.

1.3.3 Paper machine bacteria originally isolated from hot springs

From bacterial point of view paper machine resembles hot spring in many aspects: warm water is continuously running on solid surfaces. Bacteria isolated from both hot springs and paper machines are listed in Table 2.

Table 2 Species of paper machine bacteria that were first isolated from hot springs

Bacterium	Isolation from paper machines	Isolation from hot springs
Deinococcus geothermalis	Biofilms throughout the paper machine (Väisänen *et al.* 1998, Kolari *et al.* 2001, Peltola *et al.* 2008)	Agnano, Italy; Sao Pedro do Sul, Portugal (Ferreira *et al.* 1997)
Meiothermus silvanus	Headbox, slime from wire section (Kolari *et al.* 2003, Prince *et al.* 2009)	Vizela and Alcafache, Portugal (Tenreiro *et al.* 1995)
Pseudoxanthomonas taiwanensis	Headbox, slime from wire section, pulp, broke, water (Desjardins & Beaulieu 2003, Suihko *et al.* 2004, Kashama *et al.* 2009, Prince *et al.* 2009)	Chi-ban, Taiwan (Chen *et al.* 2002b)
Rubellimicrobium thermophilum	Coloured slime deposits (Denner *et al.* 2006)	Yellowstone National Park, USA (A strain was isolated and its 16S rRNA gene sequenced, but the strain was not further characterized, Baumgartner *et al.* 2003)
Schlegelella aquatica	(Prince *et al.* 2009)	Tainan, Taiwan (Chou *et al.* 2006)
Tepidimonas spp.	Headbox, steel surfaces (Prince *et al.* 2009, Tiirola *et al.* 2009)	Aachen, Germany; Pingtung, Taiwan; São Pedro do Sul, Portugal (Moreira *et al.* 2000, Albuquerque *et al.* 2006, Chen *et al.* 2006)
Thermomonas hydrothermalis	Kaolin slurry (Busse *et al.* 2002)	São Gemil, Portugal (Alves *et al.* 2003)

1.3.4 Primary-biofilm formers in paper machines

Only some bacteria have been documented to attach and grow on a clean steel surface under paper machine conditions. Those strains may be considered as primary-biofilm formers (Blanco *et al.* 1996, Kolari *et al.* 2001, Kolari 2003). Kolari *et al*. (2001) showed that none of their 36 *Bacillus* isolates from paper machines formed biofilm as pure cultures under conditions simulating paper machine. Even so, most strains grew in biofilms in mixed cultures with the primary-biofilm former, *Deinococcus geothermalis*. The contribution of *D. geothermalis* in biofilms of paper mills was studied quantitatively by Peltola *et al*. (2008). They noticed that DNA of *D. geothermalis* was commonly present on paper machine surfaces but only as a minor component (representing <1% of total bacterial DNA) in biofilms. The authors concluded that *D. geothermalis* is a pioneer colonizer in paper machines and helps other bacteria to grow on surfaces. Kolari *et al*. (2003) isolated 95 coloured biofilm forming strains from six different paper machines. These authors

recognized four groups of primary-biofilm forming bacteria: *D. geothermalis*, *Meiothermus silvanus*, a putative new species related to *Roseomonas* and a novel genus related to *Rhodobacter* (later described as *Rubellimicrobium thermophilum* gen. nov., sp. nov. (Denner *et al.* 2006)). *Pseudoxanthomonas taiwanensis* has been reported many times from paper machines (Table 1). Raulio *et al*. (2008) showed that *Psx*. *taiwanensis* attaches and grows on a clean steel surface; more biomass accumulated with monocultures of *Psx*. *taiwanensis* than with those of *M*. *silvanus* or *D*. *geothermalis*. Tiirola *et al*. (2009) studied young biofilms with molecular methods at two different paper machines and found that the major early colonizers on a clean steel surface were *Tepidimonas* spp. at one of the machines and α-*proteobacteria* related to *Rhodobacter* spp. at the other machine. There are also three recent PhD theses discussing biofouling and primary-biofilm formers of paper machines (Kanto Öqvist 2008, Raulio 2010, Peltola 2011).

1.3.5 Taxonomy of the phylum *Deinococcus-Thermus*

Many of the primary-biofilm formers found in paper machines are members of phylum *Deinococcus-Thermus.* This phylum is one of the deep-rooted bacterial groups (Krieg & Garrity 2001), meaning that its phylogenetic distance to other phyla is large. This indicates that it has separated from the other known phyla a very long time ago. Presently it comprises two orders, *Deinococcales* (Battista & Rainey 2001c) and *Thermales* (Rainey & da Costa 2001). The order *Deinococcales* contains two families, *Deinococcaceae* (Battista & Rainey 2001a) and *Trueperaceae* (Albuquerque *et al.* 2005), each of which contains only one genus, *Deinococcus* (Battista & Rainey 2001b) and *Truepera* (Albuquerque *et al.* 2005), respectively. The *Thermales* order holds one family only, *Thermaceae* (da Costa & Rainey 2001), with five genera: *Marinithermus* (Sako *et al.* 2003), *Meiothermus* (Nobre & da Costa 2001), *Oceanithermus* (Miroshnichenko *et al.* 2003a), *Thermus* (da Costa *et al.* 2001) and *Vulcanithermus* (Miroshnichenko *et al.* 2003b).

Deinococcus is the largest genus of the phylum *Deinococcus-Thermus*. Currently (December 2010), it comprises 45 species. Most of the species were described during past decade: in 2001 the genus consisted of only seven members (Battista & Rainey 2001b). The most striking feature of this genus is its resistance to high doses of irradiation (Battista & Rainey 2001b), although some of the recently described species tolerated only moderate doses of radiation (Callegan *et al.* 2008). The genus *Deinococcus* is heterogeneous, it includes both spherical and rod shaped bacteria and some of the species stain Gram-negatively whereas others stain Gram-positively (Battista & Rainey 2001b). Most species are mesophilic

(Battista & Rainey 2001b, Rainey *et al.* 2005), but also psychrophilic (Callegan *et al.* 2008, Hirsch *et al.* 2004) and moderately thermophilic (Ferreira *et al.* 1997, Asker *et al.* 2008) species are included. 16S rRNA sequence similarities within this genus go down to 84.5% (Weon *et al.* 2007). This is low compared to many other genera. For example, the 16S rRNA sequence similarities between the species within the order *Thermaceae*, the genera *Thermus*, *Marinithermus*, *Oceanithermus* and *Meiothermus* are all above 87% (Miroshnichenko *et al.* 2003a). It is likely that genus *Deinococcus* will be divided into several genera in the future.

Family *Trueperaceae* was described based on one species, *Truepera radiovictrix*, which was isolated from a hot spring (Albuquerque *et al.* 2005). It is differentiated from the family *Deinococcaceae* by the distinct phylogenetic position, lack of detectable peptidoglycan, presence of long chain 1,2 diols and homolactic fermentation. In addition, it is alkaliphilic and slightly halophilic (Albuquerque *et al.* 2005).

1.4 Cell to cell signaling in bacteria: Quorum sensing

Quorum sensing means cell-density dependent signaling in bacterial community. Quorum sensing is often involved in biofilm formation, but it also controls many other bacterial processes, such as bioluminescence, antibiotic production and virulence factor expression (Camilli & Bassler 2006, Lopez *et al.* 2010). An autoinducer is a signaling molecule produced by a bacterial community. The extracellular concentration of the autoinducer depends on the cell density. By sensing the concentration of the autoinducer, cells get information on their population density. Sufficiently high concentration of the autoinducer results into alteration of gene expression and a community level response to high cell density.

Cell density required for the activation of quorum sensing controlled genes varies depending on environmental conditions. Dulla and Lindow (2008) found that quorum sensing activation took place faster and with smaller groups of *Pseudomonas syringae* cells on dry leaves where signal diffusion is restricted, than on wet leaves (Dulla & Lindow 2008). Recently, it was noticed that quorum sensing can occur even on single isolated *Staphylococcus aureus* cell (Carnes *et al.* 2010). It has been proposed that, in addition to cell density, bacteria also monitor other environmental properties with quorum sensing molecules, such as diffusion rates (Redfield, 2002) and pH (Decho *et al.* 2009). Quorum sensing was recently reviewed by Platt and Fuqua (2010).

The molecules responsible for transmitting quorum sensing signals vary between bacteria. Gram-negative bacteria commonly use acyl homoserine lactones, whereas Gram-positive bacteria use oligopeptide signal molecules. Autoinducer-2 (AI-2) is the only quorum sensing system that has been found both in Gram-negative and in Gram-positive bacteria (Vendeville *et al.* 2005, Camilli & Bassler 2006). Table 3 summarizes some of the quorum sensing molecules used by Gram-positive bacteria.

AI-2 was first described to control the bioluminescence of *Vibrio harveyi* (Bassler *et al.* 1994). Currently, AI-2 is used as a collective term of bacterial communication promoting molecules derived from 4,5-dihydroxy-2,3-pentanediene (DPD, Miller *et al.* 2004, De Keersmaecker *et al.* 2006). The gene encoding LuxS, the enzyme that catalyzes formation of DPD, is conserved and was found from most *Gamma*-, *Beta*- and *Epsilonproteobacteria* and *Firmicutes* (Sun *et al.* 2004). Therefore, it has been proposed that AI-2 is involved in inter-species signaling (Camilli & Bassler 2006). However, LuxS also has an important metabolic function in activated methyl cycle and thus it is not clear if all *luxS* possessing bacteria use AI-2 molecules in quorum sensing (Sun *et al.* 2004, Vendeville *et al.* 2005, De Keersmaecker *et al.* 2006,).

DPD spontaneously rearranges into AI-2 signaling molecules. Their structure has been described from two species only: *V. harveyi* (Chen *et al.* 2002) and *Salmonella enterica* serovar Typhimurium (Miller *et al.* 2004). The key molecules of AI-2 system are presented in Table 4.

Table 3. Examples of quorum sensing signal molecules in Gram-positive bacteria

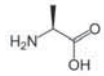
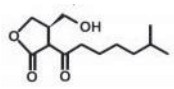
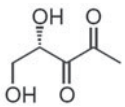
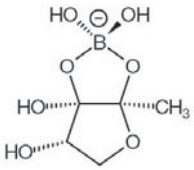
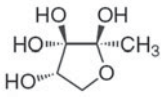
Species	Molecule	Structure of the molecule or length of the peptide	Processes controlled	Reference
Bacillus cereus	Autoinducer-2 (AI-2)	Not known (see table 4)	Inhibition of biofilm formation and promotion of cell release from preformed biofilms	Auger *et al.* 2006
Bacillus cereus	L-alanine		Spore germination	Dodatko *et al.* 2009
Bacillus subtilis	ComX	6 amino acids	Surfactin production and stimulation of natural competence	Magnuson *et al.* 1994, Okada *et al.* 2005, Lopez & Kolter 2010
Bacillus subtilis	Surfactin	7 amino acids	Biofilm formation	Lopez *et al.* 2009
Bacillus subtilis	Subtilin	32 amino acids	Subtilin production	Kleerebezem, 2004
Bacillus subtilis, B. mojavensis	CSF (PhrC)	5 amino acids	Communication between different strains which are unable to communicate with ComX	Pottathil *et al.* 2008
Lactobacillus plantarum	Plantaricin A (PlnA)	26 amino acids	Production of two-peptide bacteriocins	Diep *et al.* 2009
Lactococcus lactis	Nisin	34 amino acids	Nisin production	Kleerebezem, 2004
Staphylococcus aureus	Autoinducer-2 (AI-2)	Not known (see table 4)	Capsular polysaccharide production	Zhao *et al.* 2010
Staphylococcus spp.	Autoinducing peptides (AIP I-IV)	7-9 amino acids	Expression of virulence and other accessory genes	Novick & Geisinger, 2008
Streptococcus spp.	Competence stimulating peptide (CSP)	16 amino acids	Development of competence and biofilm formation	Petersen *et al.* 2004, Suntharalingam & Cvitkovitch 2005
Streptococcus spp.	Autoinducer-2 (AI-2)	Not known (see table 4)	Biofilm formation and antibiotic resistance	Ahmed *et al.* 2007, Ahmed *et al.* 2008
Streptomyces griseus	A-factor		Streptomycin production	Ohnishi *et al.* 1999

Table 4. Key molecules in autoinducer-2 based quorum sensing

Function	Name	Structure	Reference
Enzyme catalyzing formation of 4,5-dihydroxy-2,3-pentanediene (DPD)	LuxS		Surette *et al.* 1999
Precursor of AI-2 signaling molecules	DPD		Schauder *et al.* 2001, Camilli & Bassler 2006,
AI-2 from *Vibrio harveyi*	S-THMF-borate		Chen *et al.* 2002
AI-2 from *Salmonella enterica* serovar Typhimurium	R-THMF		Miller *et al.* 2004, Camilli & Bassler 2006

1.5 Biofilm mode of growth

Biofilms are multicellular communities held together by a self-produced extracellular matrix (Lopez *et al.* 2010). Biofilms have been studied extensively during past three decades, and there are numerous reviews discussing different aspects of biofilm mode of growth (for example: Costerton *et al.* 1995, Costerton *et al.* 1999, Hall-Stoodley *et al.* 2004, Ghannoum & O'Toole 2004, Costerton 2007, Romeo 2008, Lopez *et al.* 2010). Biofilm formation is an ancient property of micro-organisms and it is common for both *Bacteria* and *Archaea* (Hall-Stoodley *et al.* 2004). Biofilm cells are held together by an extracellular matrix. Its composition varies greatly between bacterial species and environmental conditions. Its best known constituents are polysaccharides and proteins (Branda *et al.* 2005, Flemming & Wingender 2010, Abee *et al.* 2010), but also extracellular DNA is an important component of biofilms (Das *et al.* 2010). Biofilm production depends on environmental conditions. Different species and even strains of one species use different molecular mechanisms to regulate biofilm formation (Lopez *et al.* 2010). Most natural biofilms harbour many bacterial species (Kolter & Greenberg 2006).

Growing in biofilm is beneficial for bacteria in many ways. Probably the most important of them is the protection against various threats. Biofilm protects the bacteria against protozoa, host defences and many antibiotics (Stewart *et al.* 2004, Lewis 2008, Lopez *et al.* 2010). Attached cells are resistant to physical forces and can remain on a favourable environment without being washed away by liquid flow (Jefferson, 2004). In addition, lateral gene

transfer is faster in biofilms where the cells are closer to each other than are planktic cells, which can be an advantage (Hausner & Wuertz 1999, Jefferson 2004).

1.5.1 *Bacillus* biofilms

Among the species of the genus *Bacillus*, biofilm formation is most studied with *B. subtilis*. It forms floating pellicles with complex structure when grown in stationary conditions (Branda *et al.* 2001). The extracellular matrix of *B. subtilis* mainly consists of a protein component TasA and a polysaccharide component (Branda *et al.* 2006). The genes involved in biofilm formation of *B. subtilis* have been extensively studied and there are recent reviews discussing the biofilm formation of *B. subtilis* (Lemon *et al.* 2008, Lopez *et al.* 2010).

Recently, it was noted that compounds causing K^+ leakage triggered biofilm formation in *Bacillus subtilis* (Lopez *et al.* 2009). Surfactin, a cyclic lipopeptide produced by *B. subtilis* itself, was one of the compounds. Already earlier it was noticed that a surfactin deficient mutant of *B. subtilis* did not form fruiting bodies (Branda *et al.* 2001) and that surfactin deficient mutant strains produced less biofilm than the wild type (Bais *et al.* 2004, Hofemeister *et al.* 2004). Lopez *et al*. (2009) noticed that surfactin induced transcription of *epsA-O* and *yqxM-sipW-tasA*, the two multigene operons responsible for the matrix production. They also noticed that this activation happened only in a subpopulation of the cells. Membrane histidine kinase KinC was involved in the induction of the transcription of these genes by phosphorylating the master regulator Spo0A. In addition, the same authors noticed that surfactant properties of surfactin were not required for the biofilm formation and that addition of 150 mM of K^+, but not of lithium or of sodium ions, inhibited the ability of surfactin to induce biofilm formation. Thus, the authors concluded that surfactin acted as an autoinducer and loss of K^+ ions was the signal to activate the membrane protein kinase KinC, which led into formation of the biofilm.

B. subtilis cells differentiate into distinct subpopulations during biofilm formation. Only a fraction of the cells produce the signaling molecule surfactin whereas the other cells specialize in matrix production or differentiate into miners (secreting proteases (Veening *et al.* 2008)), cannibals (producing protein toxins Skf and Sdp, which kill neighbouring cells (Gonzalez-Pastor *et al.* 2003)), motile cells or spores. This differentiation is possible because of paracrine signaling, meaning that some cells produce signaling molecules and only a part of the population responds to this (Lopez *et al.* 2009, Lopez & Kolter 2010).

1.5.2 *Bacillus cereus* biofilms

Wijman *et al*. (2007) studied biofilm formation with 56 strains of *B. cereus* and found that thick biofilm developed at the air-liquid interphase on the walls of polystyrene microplates. Biofilm forming abilities of the strains varied greatly. The defined medium Y1 supported biofilm growth much more than did LB-medium (54 and 21 biofilm forming strains out of 56, respectively). Biofilm formation also depended on the incubation time (more biofilm in 24 h than in 48 h). The type strain ATCC 14579^T formed biofilm only in Y1 medium and the biofilm was visible after 24 h incubation, but disappeared by 48 h (Wijman *et al.* 2007). Auger *et al*. (2009) compared the biofilm forming properties of 102 strains of *B. cereus*. They found that strains of *B. thuringiensis* (n=24) and nonclinical (n=30) and diarrheal (n=16) strains of *B. cereus* frequently (35-45% of the strains but not the type strain ATCC 14579^T) produced biofilm in LB-medium at 30°C on PVC microplates. Instead, none of the emetic (n=20) or oral (n=12) isolates formed biofilm under these conditions (Auger *et al.* 2009). Hsueh *et al*. (2008) studied biofilm formation of 22 strains of different origins in low (EPS-medium) and high (LB) nutrient medium at 32°C. All but two strains formed biofilm in EPS-medium (12 h incubation) and most strains produced more biofilm in EPS-medium than in LB (Hsueh *et al.* 2008).

Some work has been done to reveal the molecular mechanisms behind the formation of biofilm in *B. cereus*. Cell wall peptidase CwpFM was shown to be involved in biofilm formation (Tran *et al.* 2010). Compared to the wild type, a *cwpFM* mutant adhered poorly to HeLa cells and formed less biofilm on PVC microplate (LB-medium, 48 h, 30°C). Hsueh *et al*. (2008) noticed that *codY* mutants, which had three times higher protease activity than the wild type, produced four times less biofilm than the wild type. They also noticed that proteinase K inhibited biofilm formation of *B. cereus*. Earlier, same authors (Hsueh *et al.* 2006) had noticed that the pleiotropic regulator PlcR influenced biofilm formation of *B. cereus* strain ATCC 14579^T. Mutant Δ*plcR* produced more biofilm and increased amounts of biosurfactant compared to the wild type. Also, added surfactin (biosurfactant from *B. subtilis*) increased the biofilm formation of the wild type.

Auger *et al*. (2006) showed that cell-free supernatant of *B. cereus* strain ATCC 10987 induced luminescence in a *Photorhabdus luminescens ΔluxS* mutant. This indicates that *B. cereus* ATCC 10987 produced functional quorum sensing signal AI-2. The induction of luminescence was highest with supernatant from 8 h grown culture (LB-medium, 37°C, 200 rpm). Synthetic DPD (precursor of AI-2 molecules) inhibited biofilm formation and

promoted the release of cells from preformed biofilms of *B. cereus* ATCC 10987 (Auger *et al.* 2006). Andersson *et al*. (2007) noticed that ethanol extracts of *B. cereus* strains (F4810/72, GR177, NS61 and P113) induced light emission of *V. fischerii*. The induction, up to 600 %, may indicate production of quorum sensing molecules by these *B. cereus* strains.

Shi *et al*. (Shi *et al.* 2004) showed that genes involved in the metabolism of polyphosphates (*ppk*, *ppx* and *pap*) affected also biofilm formation. Mutant strains Δ*ppk*, Δ*ppx* and Δ*pap*, which were also defective in swimming and swarming, produced less biofilm than the wild type strain ATCC 14579^T. Houry *et al*. (2010) studied the role of flagelli and motility on the biofilm formation with *B. cereus* strain 407. In standing cultures, the non-motile mutants *Δfla* (non-flagellated) and *ΔmotA* (flagellated but non-motile) formed less biofilm at the air-liquid interphase of PVC and glass surfaces. In flow cells mutants produced same amount of biofilm as the wild type. The mutant *Δfla* attached even more efficiently than the other strains. The authors concluded that in standing cultures motility was necessary for reaching suitable surfaces at the air-liquid interphase, but in the flow cell bacteria could reach the surface through sedimentation (Houry *et al.* 2010). Vilain *et al*. (2009) showed that three *B. cereus* mutants deficient in purine biosynthesis genes (*purA*, *purC*, and *purl*) formed less biofilm than the wild type ATCC 14579^T (LB, 72 h, static at 25°C). Extracellular DNA (eDNA) was detected from the wild type cells in exponential growth phase and from the EPS of the biofilm. Vilain *et al*. (2009) concluded that eDNA is an integral component of the EPS of *B. cereus* ATCC 14579^T biofilms.

1.6 Potassium homeostasis in bacteria

In natural environments K^+ is usually scarce whereas Na^+ is abundant. Instead, K^+ is the main cation in the cytoplasm of all living cells, present in concentrations much higher than Na^+. K^+ contributes to electrical neutralization of anionic groups and basic physiological functions, such as controlling the electric potential of the cell membrane and the osmotic balance (Corratge-Faillie *et al.* 2010). The reason why cytosol is rich in K^+ instead of Na^+ may be that kosmotrophic Na^+ ions are strongly hydrated in aqueous solutions whereas K^+ ions are chaotrophic and thus only weakly hydrated with no tightly bound molecules of water (Collins *et al.* 2007). Kosmotrophic cations, such as Na^+, also destabilize proteins whereas chaotrophic cations, like K^+, stabilize them, as reviewed by Zhao (2005). For the primitive cell that lived in seawater more than three billion years ago, accumulation of K^+ and exclusion of Na^+ may have been the primordial way to energize the plasma membrane (Corratge-Faillie *et al.* 2010).

1.6.1 Potassium uptake in bacteria

Bacteria have diverse potassium transporters to maintain the necessary cytoplasmic K^+ concentration. K^+ specific ABC transporter Kdp-ATPase, which pumps K^+ ions into cell utilizing the energy from ATP hydrolysis, is a well studied potassium transporter. It is conserved among many bacterial species, including *B. cereus* strain E33L (Ballal *et al.* 2007). The K_m (substrate concentration where reaction rate is half of the maximal rate) of Kdp-ATPase was reported as 25 µM K^+ in *Rhodobacter sphaeroides* (Abee *et al.* 1992). Kdp-ATPase has high affinity towards K^+, but rate of K^+ uptake is low (Corratge-Faillie *et al.* 2010). Therefore, bacteria have also other means for K^+ uptake, which operate at higher rate, but have lower affinity. K^+ import is coupled to import of H^+ ions with transporters belonging to family Trk and to import of Na^+ ions with transporters belonging to Ktr family (Corratge-Faillie *et al.* 2010).

Potassium specific ion channels are conserved among living organisms and are found from both bacteria and eukaryotes (MacKinnon *et al.* 1998). The detailed structure of a K^+ channel from *Streptomyces lividans* has been described (Doyle *et al.* 1998) and Roderick MacKinnon was later awarded with a Nobel prize (2003) for these findings. Nevertheless, the function of K^+ selective channels in prokaryotes is poorly understood and it is not known whether they participate to the uptake of K^+ or not (Kuo *et al.* 2005)..

1.6.2 Microbially synthetized potassium ionophores

Microbes produce two types of potassium ionophores, *i.e.* lipid soluble molecules which can transport K^+ across biological membranes. Pore forming molecules (*e.g.* alamethicin and gramicidin (Gräfe, 1992)) make channels through cell membrane and K^+ ions can diffuse through them. Carrier molecules, such as valinomycin, enniatine, nigericin, salinomycin, lasalocid A (Gräfe, 1992), and cereulide (Agata *et al.* 1994) bind potassium ions and transport them across the membrane. Both of these transport mechanisms are driven by electrochemical gradient across the membrane. Many of these compounds have been shown to be antibacterial in micromolar concentrations (Leitgeb *et al.* 2007), but the biological benefit of these molecules for the producer organisms is unclear.

Table 5. Examples of microbially produced potassium ionophores

Substance	Producer organism	Reported antimicrobial activity	References
Alamethicin	*Trichoderma* spp.	Inhibition of Gram-positive bacteria (15 – 60 µM in BHI).	Meyer & Reusser 1967, Leitgeb *et al.* 2007
Gramicidin	*Bacillus brevis*	Growth inhibition of *Streptococcus faecalis* (0.1 µM in medium with low [K^+]).	Harold & Baarda 1967
Valinomycin	*Streptomyces* spp.	Inhibition of Gram-positive bacteria in disc diffusion assay (Minimum inhibitory concentration 0.4 - 0.7 nmol/disc). Growth inbibition of *S. faecalis* (1 µM in medium with low [K^+]).	Harold & Baarda 1967, Pettit *et al.* 1999, Kroten *et al.* 2010
Enniatine	*Fusarium* spp.		Tonshin *et al.* 2010
Salinomycin	*Streptomyces albus*	Used for controlling dysentery and *Clostridium perfringens* infections in pigs.	Butaye *et al.* 2003
Cereulide	*Bacillus cereus*	170 nM reduced light emission of *Vibrio fischerii* by 50 % in 30 min contact time. 9 µM inhibited growth of Gram-positive bacteria (BHI, pH 8.5), excluding cereulide producing strain F4810/72.	Agata *et al.* 1994, Agata *et al.* 1995, Andersson *et al.* 2007, Kroten *et al.* 2010, Tempelaars *et al.* 2010

1.7 Cereulide, the emetic toxin of *Bacillus cereus*

Cereulide is the toxin causing vomiting disease in food poisonings connected to *B. cereus*. Cereulide molecule is a cyclic depsipeptide of 1.2 kDa consisting of 6 amino and 6 hydroxy fatty acid residues (Agata *et al.* 1994, Agata *et al.* 1995) (Figure 1). This toxin is heat stable (not inactivated by autoclaving (Shinagawa *et al.* 1996, Rajkovic *et al.* 2008)) and highly hydrophobic (log K_{ow} 6.0 (Teplova *et al.* 2006)). The affinity of cereulide towards K^+ ions is higher than that of any other known K^+ carrier, including structurally similar compound valinomycin (Teplova *et al.* 2006, Makarasen *et al.* 2009), and it binds K^+ ions in concentrations below 1 mM (Teplova *et al.* 2006). It affects on the membrane potential and K^+ homeostasis of mammalian cells and mitochondria (Jääskeläinen *et al.* 2003, Teplova *et al.* 2006). Mikkola *et al*. (1999) found that the conductance of lipid membranes towards K^+ ions was increased by cereulide three orders of magnitude more than the conductance towards Na^+ or to NH_4^+. These data indicate that cereulide is highly selective for potassium.

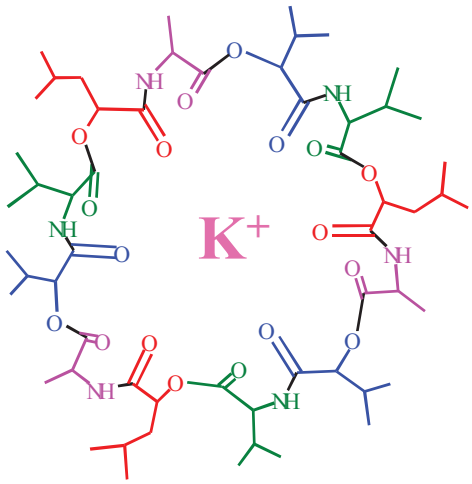


Figure 1. Structure of cereulide. The molecule consists of three repeats, each containing two hydroxy and two amino acids (D-O-Leu-D-Ala- L-O-Val-L-Val)3 (www.biocenter.helsinki.fi /groups/salkinoja/index.htm).

1.7.1 Genetic background of cereulide synthesis

Cereulide is synthesized by a nonribosomal peptide synthetase (NRPS, Horwood *et al.* 2004, Toh *et al.* 2004, Ehling-Schulz *et al.* 2005). The gene cluster *ces* (*cesA*, *cesB*, *cesC*, *cesD*, *cesH*, *cesP* and *cesT*) is responsible for cereulide synthesis. The *cesH*-gene is transcribed from its own promoter, the other *ces*-genes are transcribed as a single large (23 kb) transcript (Dommel *et al.* 2010). The *ces* cluster is located on a plasmid pCER270 (also known as pCERE01). The 270 kb plasmid has sequence similarity to the virulence plasmid of *Bacillus anthracis,* pXO1 (Hoton *et al.* 2005, Ehling-Schulz *et al.* 2006, Rasko *et al.* 2007). Later Hoton *et al*. (2009) noticed that the size of the cereulide biosynthesis coding plasmid varied between 240 and 290 kb and they also found five isolates of *B. weihenstephanensis* producing cereulide but possessing no pXO1-like plasmid. The genes for cereulide synthesis were located on another plasmid (300 – 320 kb, two strains), or in the chromosome or on a very large plasmid (three strains) (Hoton *et al.* 2009).

Lücking *et al*. (2009) showed that the master response regulator Spo0A (but not the pleiotropic regulator PlcR, which regulates most virulence factors of *B. cereus*) plays an important role in cereulide synthesis, since *spoOA* null mutant produced no cereulide. Spo0A promotes cereulide synthesis by down-regulating transcription factor ArbB, which binds to the promoter region of the *ces* operon and thus may directly suppress cereulide synthesis. Although SpoOA is also involved in the regulation of sporulation, it seems that sporulation and cereulide production are independent processes since it was reported that cereulide synthesis started long time before sporulation (Häggblom *et al.* 2002), and a toxic but non-sporulating mutant was found (Lücking *et al.* 2009).

1.7.2 Effects of environmental conditions on cereulide production

Several studies have been published concerning the environmental conditions affecting cereulide production (Table 6). The results vary and it is clear that cereulide production is affected by multiple environmental signals. Some general trends can be seen, though.

Under nitrogen atmosphere (>98%) cereulide production has not been observed (Jääskeläinen *et al.* 2004, Rajkovic *et al.* 2006a). This is not because of lack of oxygen since high amount of cereulide was produced in anaerobic atmosphere containing 9-13% carbon dioxide (Jääskeläinen, 2008). It is not clear how aeration of liquid cultures by shaking affects the production of cereulide.

In general, more cereulide was produced on rich media (TSA, Blood agar, BHI-agar) than on poorer ones (R2A, MYP-agar, rice water agar) (Jääskeläinen *et al.* 2004, Rajkovic *et al.* 2006a, Apetroaie-Constantin *et al.* 2008), although Dommel *et al*. (2010) found that cereulide synthetase promoter activity was the lowest on TSA of all 9 media used.

No cereulide production was reported below 12°C or above 40°C, although many cereulide producing strains grow outside these temperature limits. In most studies, highest cereulide yields have been observed at 15-25°C (Szabo *et al.* 1991, Finlay *et al.* 2000, Häggblom *et al.* 2002, Thorsen *et al.* 2006, Apetroaie-Constantin *et al.* 2008).

Table 6. Effect of growth conditions of *B. cereus* on cereulide production

Cereulide producing strains used	Growth conditions			Observed effect of growth media, atmosphere and temperature	Reference
	Medium	t (°C)	Time (h)		
NS58, F4810/72, NC7401, LMG17604, RIVM BC00067, RIVM BC00075, UB1020	TSA, Blood agar, Skim milk agar, Raw milk agar, MacConkey agar, Oatmeal agar	20-23	48	Cereulide production was highest on TSA. No clear effects were seen, but after stepwise logistic regression analysis high [Na^+], [K^+]:[Na^+] and [glycine] were associated with high cereulide production. On TSA cereulide production was highest at 22°C and decreased when temperature was raised. On oatmeal agar temperature (from 22 to 35°C) had no effect.	Apetroaie-Constantin *et al.* 2008
F4810/72, B116, B203	TSB, boiled kidney beans	21	65	Cereulide production on rich media (Blood agar, BHI and TSA) was higher than on media with lower nutrient concentrations (MYP, R2A, rice water agar). Addition of free amino acids L-leucine and L-valine into R2A and rice water agar increased cereulide production 10 to 20 fold. High atmospheric nitrogen concentration (> 99.5%) suppressed cereulide production.	Jääskeläinen *et al.* 2004
B116, B203, F4810/72	TSA, TSB and consumer skim milk	22	96	Cereulide was produced 100 times more in TSB than in milk. Ambient air and anaerobic conditions resulted in same cereulide production on TSA when anaerobic atmosphere contained 9-13 % CO_2.	Jääskeläinen 2008
F4810/72, NC7401, F5881	TSB	21	24 or 70	Shaken (150 rpm) cultures yielded high cereulide concentrations, whereas static ones none or only minute amounts. High amount was produced at 21°C, whereas only very little at 11, 40 and 42°C	Häggblom *et al.* 2002
5964a, NS117	Ready-to-eat foods: potato puree, pasta, rice, bechamel sauce, milk	28	12-48	Higher amounts were detected in potato puree, pasta and rice than in milk. No cereulide was detected in the béchamel sauce. Shaking (1:5 dilution with peptone-saline (NaCl) -solution) decreased cereulide production 14-15 fold in all foods.	Rajkovic *et al.* 2006b
5964a, NS117	Solid media: PDA, milk agar, NA and TSA.	30	24	Highest amount of cereulide was produced on TSA, lowest on NA. In BHI, cereulide production occurred faster at pH 7.4 than at 6.8 or 6.0	Rajkovic *et al.* 2006a

	Liquid medium: BHI			No cereulide was produced in N_2 atmosphere containing 1.6 or 0.7% O_2 on TSA. When O_2 concentration was 4.5 or 10.6%, cereulide was produced similarly or slightly more than in ambient air. No cereulide production was observed in shaken cultures of BHI. No cereulide production was observed at 12°C in BHI (6 d)	
NC7401	Different types of foods	30	24	Highest amounts of cereulide were produced in fried or boiled rice, milk and soy milk. No cereulide was produced in egg and meat products. High amount of cereulide was produced in shaking and only little in stationary culture in milk and soy milk. More cereulide was produced at 35°C than at 20°C or 30°C. Cereulide production also started faster at 35°C.	Agata *et al.* 2002
F4810/72, NS117, NC7401, F3080B/84, F5881,RIVM-BC68,B203, *B.weihen-stephanensis* MC67 and MC118	BHI-agar		240	Cereulide production was much higher (50 – 1500 fold) at 25°C than at 15°C or 12°C.	Thorsen *et al.* 2006
F4810/72	Dairy and cereal based infant foods	21-23	24	More cereulide (up to 1000 fold) was produced in cereal based than in dairy based infant foods. Dilution of cereal containing food with water increased cereulide production. More cereulide was produced in stationary than shaken (60 rpm) infant foods.	Shaheen *et al.* 2006
F4810/72, F3748/75, F3744/75, F4562/75, F4552/75, F2427/76, F2549A/76	Skim milk medium	30	24	No heat stable toxin was produced under anaerobiosis, whether shaken or not. High toxicity was detected in shaken cultures in aerobic and microaerobic conditions. Static cultures yielded ten fold lower toxin titres than shaken. Higher toxicity was detected at 12°C (10 d incubation) and 15°C (4 d) than at 30°C or 37°C (1 d). No toxicity was detected above 37°C. Temperatures between 15°C and 30°C were not tested.	Finlay *et al.* 2000, Finlay *et al.* 2002b

F3748/75, F4552/75, F4562/75	Boiled rice		96	Higher toxicity was detected at 15°C than at 20°C or 30°C	Finlay *et al.* 2002a
F4810/72	11 different foods and culture media	27	18	Highest toxicities were detected in milk and white rice. Highest toxicities were detected from foods incubated at 25-30°C. Only minor amounts of toxin were produced at 15 or 40°C. Stationary growth conditions yielded lower toxicity titers than shaken.	Szabo *et al.* 1991
F4810/72 and F4810/72 pMDX[P_1/ *luxABCDE*]	9 solid media and 7 foods	24	24	Highest cereulide production was in béarnaise sauce, liver sausage and cooked rice. Cereulide synthetase promoter activity and produced cereulide corresponded. Highest promoter activity was on MYP, high activities were also observed on PCA, LB, BCM and fortified nutrient agar. TSA had lowest promoter activity of all tested media.	Dommel *et al.* 2010

TSA, Tryptic soy agar; PDA, Potato dextrose agar; BHI, Brain heart infusion; LB, Luria-Bertani; TSB, Tryptic soy broth; MYP, Mannitol egg yolk polymyxin; PCA, Plate count agar; BCM, *Bacillus cereus* group plating medium

1.7.3 Timing of cereulide synthesis during *B. cereus* growth

Cereulide yields at different time points of *B. cereus* growth have been measured in several studies. Cereulide becomes detectable soon after the exponential growth phase has ended and subsequently accumulates reaching highest concentration after 1-3 d of incubation at mesophilic temperatures (Häggblom *et al.* 2002, Finlay *et al.* 2000, Finlay *et al.* 2002a, Finlay *et al.* 2002b, Thorsen *et al.* 2009). The transcription of the peptide synthetase genes responsible for cereulide synthesis is growth phase dependent and *ces* promoter activity was high only 4 hours reaching highest value after 15 h growth in LB-medium at 30°C (Lücking *et al.* 2009).

1.7.4 Occurrence of emetic strains in the environment

Cereulide producing strains are rare in the natural environment. For example, among 1748 isolates from soil in four studies carried out in different countries and only 3 cereulide producing strains were found (Hoton *et al.* 2009, Thorsen *et al.* 2006, Svensson *et al.* 2006, Altayar & Sutherland 2006, Table 7). More cereulide producers were found from used bedding at dairy farms, potato skin, spruce tree, moisture damaged buildings and from mammals. In addition, many cereulide producing strains were isolated from foods, especially from foods connected to food poisonings (Table 7).

Table 7. Occurrence of cereulide producing strains in different environments

Source of the strains	Number of isolates		Proportion of cereulide producing strains (%)	Reference
	Total	Cereulide producing		
Soil (dairy farm, Sweden)	374	0	<1	Svensson *et al.* 2006
Feed (dairy farm, Sweden)	43	0	<3	Svensson *et al.* 2006
Grass (dairy farm, Sweden)	19	0	<5	Svensson *et al.* 2006
Dung (dairy farm, Sweden)	44	0	<3	Svensson *et al.* 2006
Rinsing water (dairy farm, Sweden)	339	4	1.2	Svensson *et al.* 2006
Used bedding (dairy farm, Sweden)	204	8	3.9	Svensson *et al.* 2006
Air (dairy farm, Sweden)	12	0	<8	Svensson *et al.* 2006
Milk (dairy farm, Sweden)	722	11	1.5	Svensson *et al.* 2006
Dairies (processing lines and silo tanks, Sweden)	3911	44*	1.1	Svensson *et al.* 2006
Soil, cow and horse faeces (UK)	196	0	<1	Altayar & Sutherland 2006
Potato skin (UK)	25	4	16	Altayar & Sutherland 2006
Vegetables and potato powder (UK)	84	0	<2	Altayar & Sutherland 2006
Interior of potatoes (surface sterilized)	11	6	55	Hoornstra 2008
Soil	543	1	0.2	Hoton *et al.* 2009
Insects and isopods	58	0	<2	Hoton *et al.* 2009
Mammals	109	18	17	Hoton *et al.* 2009
Commercial wastewater powder	57	0	0	Hoton *et al.* 2009
Foods (random samples)	582	8	1.5	Hoton *et al.* 2009
Soil (sandy loam, Denmark)	390	2	0.5	Thorsen *et al.* 2006
Soil (curly kale fields, Denmark)	245	0	<1	Thorsen *et al.* 2006
Leaves of curly kale (Denmark)	286	0	<1	Thorsen *et al.* 2006

Norway spruce (total number of *B. cereus* isolates was 27, six were tested for cereulide production, Finland)	6	4	66	Hallaksela *et al.* 1991, Shaheen 2009
Indoor air, dust and building materials from moisture damaged buildings (Finland)	20	8	40	Andersson *et al.* 2005
Foods, not connected to illness	144	35	24	Shaheen 2009
Foods, connected to food poisonings	86	25	29	Shaheen 2009
Pasta and meat dish, connected to food poisoning (Finland)	122	83	68	Pirhonen *et al.* 2005

*40 of the strains were isolated from a single silo tank

2. Aims of the study

The aim of this study was to widen the understanding of microbes involved in biofouling of paper machines. The specific aims were:

1. To identify bacteria colonizing paper machines (Papers I and III, this thesis).
2. To assess the role of bacteria in the formation of end product defects during the paper making (Paper I, this thesis).
3. To assess the quantitative contribution of *Meiothermus* spp. into biofilms of the wet-end of paper machine (Paper I, this thesis).
4. To quantify the transfer of *Bacillus cereus* spores from packaging board into food (Paper II).
5. To find out how the producer organism benefits from cereulide, the food poisoning toxin produced by certain *Bacillus cereus* strains (Paper IV, this thesis).

3. Materials and Methods

Methods used in this thesis work are listed in Table 8. Most of the methods are described in detail in Papers I-IV. Methods not used in the publications are described here.

Table 8. Methods used during this thesis work

Method	Described in Paper
DNA extraction	
From pure cultures	I
From industrial process samples	I, Kanto Öqvist *et al.* 2008
16s rRNA gene sequencing	I, III
Quantitative PCR	
Universal bacterial primers	I
Primers specific for the genus *Meiothermus*	I
Primers specific for *Pseudoxanthomonas taiwanensis*	This thesis
Quantification of bacterial biomass using ATP measurement	This thesis
Incorporating fluorescently labelled spores in paper	II
Quantification of spore transfer from paper to food	II
Isolation of bacterial pure cultures	III
Characterization of bacterial isolates	
Analysis of optimal growth temperature	III
Analysis of optimal growth pH	III
Analysis of optimal salt concentration for growth	III
Oxidation of carbon sources and utilization nitrogen, phosphorus and sulphur sources using Biolog Phenotypic MicroArray	III
Production of acid and gas from carbohydrates	III
Oxidase test	III
Nitrate reduction test	III
Radiation resistance test	III
Whole cell fatty acid analysis	III
Phylogenetic analysis	III
Biofilm assay	IV
Use of the membrane potential sensitive dye JC-1 for bacteria	This thesis
Pellicle formation test	IV, this thesis
Bacterial competition assay	IV
Growth rate measurement	IV
K^+ leakage measurement	IV

3.1 Strains used in this study

The strains used in the development of qPCR method for *Meiothermus* spp. are listed in the Table 1 of Paper I. The reference strains used in the description of genus *Deinobacterium* are listed in Table 1 of Paper III, and the strains used for exploring the biological function of cereulide are listed in the Materials and Methods section of Paper IV. Strains used in the development of qPCR method for *Pseudoxanthomonas taiwanensis* are listed in Table 9.

Table 9. Strains used in development of qPCR method for *Pseudoxanthomonas taiwanensis*

Strain
Pseudoxanthomonas taiwanensis JN11003[1, 3]
Pseudoxanthomonas sp. JN41003[1, 3]
Pseudoxanthomonas sp. S2-bf-PMWA-8[1, 3]
Pseudoxanthomonas broegbernensis DSM 12573
Porphyrobacter cryptus A-col-BFA6[1, 3]
Thermomonas haemolytica DSM 13605[T]
Thermomonas hydrothermalis JN31003[1, 3]
Staphylococcus epidermidis DSM 20044[T]
Stenotrophomonas maltophilia DSM 50170[T]
Xanthomonas campestris DSM 3586[T]
Deinococcus geothermalis E50051[2, 3]
Pseudomonas boreopolis S2-s-PMWA-6[2, 3]
Meiothermus silvanus B-R2A5-50.4[2, 3]
Escherichia coli MT102[2]

[1]Identification based on 16S rRNA gene sequence
[2]Paper I
[3]Paper machine isolates from the culture collection of Prof. Salkinoja-Salonen

3.2 Quantitative PCR method for *Pseudoxanthomonas taiwanensis*

The primers specific to the 16S rRNA gene of *Pseudoxanthomonas taiwanensis* (Table 10) were constructed using the ARB program package (Ludwig *et al.* 2004). Quantitative PCR was done using LightCycler quantitative real-time PCR machine (Roche Diagnostics, Penzberg, Germany) and SYBR Premix Ex Taq –reagent (Takara Bio Inc., Shiga, Japan). The reaction volume (20 µl) consisted of DNA sample (2 µl), 0.3 µM primers and 10 µl SYBR-reagent. The PCR program was as follows: 30 s at 95°C followed by 40 cycles of 10 s at 95°C and 20 s at 60°C. After the amplification, the melting temperatures of the

amplification products were analyzed by raising the temperature from 63°C to 98°C, 0.1°C per second.

Table 10. Primers used for quantitative PCR in this study

Target organisms	Primer	Sequence (5' → 3')	Position*	Reference
Psx. taiwanensis	PsxF-829	ATG TTG GGT TCA ATT TGG GAC	830 - 850	This thesis
	PsxR-1017	TCC CGA AGG CAC CCG CCC	1035 - 1018	This thesis
Meiothermus spp.	MeioF692	GAA ATG CGC AGA TAC CGG A	692 - 711	Paper I
	MeioR821	TGT CGG ACA CCC AGC ACT	821 - 839	Paper I
Eubacteria	pE	AAA CTC AAA GGA ATT GAC GG	906 - 926	Edwards *et al.* 1989
	pF'	ACG AGC TGA CGA CAG CCA TG	1073 - 1053	Edwards *et al.* 1989

*Based on *E. coli* numbering

3.3 Quantification of bacterial biomass using ATP measurement

Amount of ATP was quantified with ATP Biomass Kit HS (BioThema AB, Handen, Sweden) according to the manufacturer's instructions and converted into bacterial cell numbers assuming the ATP content as 2×10^{-18} mol/cell.

3.4 Detection of bacterial membrane potential with JC-1 staining

The bacteria were grown in TSB medium. Cells from exponential growth phase were harvested by centrifugation (5 min 3000 rpm), washed with 1 mM Na-phosphate buffer (pH 7.3) and finally suspended into the phosphate buffer. The cell suspension was divided in three aliquots, of which the first was supplemented with 120 mM KCl, the second with 5 mM glucose and the third with none. Each aliquot was divided into two portions of which one was treated with 70 µM cereulide (cereulide containing extract in methanol) and the other with same volume methanol. The cell suspensions were stained with the membrane potential sensitive fluorescent dye JC-1 and results observed with a fluorescence microscope.

3.5 Pellicle formation assay

Actively growing cells (in TSB) were diluted ten fold with sterile TSB medium. The cell suspensions were dispensed into the wells of a polystyrene microplate (200 µl/well). Cereulide containing extract (in methanol) was added to 10 µM cereulide or same volume methanol into the vehicle control wells. The plate was incubated at 28°C (shaking 120 rpm) for 17 h after which it was photographed.

For phase-contrast images, overnight cultures of *B. cereus* strains (in TSB) were diluted ten fold with sterile drinking water. The cell suspensions were dispensed into wells of a polystyrene microplate (200 µl/well) and cereulide was added (10 µM or same volume methanol into the vehicle control wells). The plate was incubated 23 h (28°C, 120 rpm) and the results were observed with phase-contrast microscopy.

4. Results and Discussion

4.1. Bacteria in paper machines

4.1.1. *Meiothermus* spp. are major biofoulers in paper machines

I found *Meiothermus* DNA in biofilms obtained from 18 paper machines. This was 75% of the studied machines (Table 4 of Paper I). PCR data indicate that high numbers of *Meiothermus* spp. were or had been present in the biofilms, especially in those from headbox, wire section and disc filters. *Meiothermus* 16S rRNA genes occurred in densities up to 10^{11} /g wet weight (Table 4 of Paper I) in samples collected from paper machines in Finland, Sweden, China and USA. In addition to my results, Prince *et al*. (2009) found using DNA methods that *Meiothermus* was the most common genus in slurry of the headbox of a Canadian paper mill. Earlier, Kolari *et al*. (2003) isolated 95 pigmented biofilm forming strains and identified 25 of these as *Meiothermus* spp.. They were found in 5 out of the studied 6 paper, board and pulp mills. *M. silvanus* was the most common species with 18 isolates (Kolari *et al.* 2003). These findings show that *Meiothermus* spp. are widespread in paper machines around the world.

Currently (January 2011), the genus *Meiothermus* comprises of nine species, *M. ruber*, *M. silvanus*, *M. chliarophilus*, *M. cerbereus*, *M. taiwanensis*, *M. timidus*, *M. rufus*, *M. cateniformans* and *M. granaticius.* All of these species were first described from hot springs (Loginova *et al.* 1984, Tenreiro *et al.* 1995, Chung *et al.* 1997, Chen *et al.* 2002a, Pires *et al.* 2005, Albuquerque *et al.* 2009, Albuquerque *et al.* 2010, Zhang *et al.* 2010). At the time of submission of the Paper I describing the qPCR method for *Meiothermus* spp. (June 2006), only five of these nine species had been validly described and thus only their sequences were used for method development. However, according to BLAST, the primers developed in Paper I (MeioF692 and MeioR821, Table 10) represent a complete match also with the 16S rRNA genes of the species *M. timidus*, *M. rufus* and *M. cateniformans* that were described later. Thus, the DNA of also these species will most likely be amplified with our qPCR method. One recently described species, *M. granaticius* (Albuquerque *et al.* 2010) has 2 mismatches with the forward primer MeioF692 and one with the reverse primer MeioR821, indicating poor quantification result.

When the qPCR method for the genus *Meiothermus* was developed, the accurate genome size and the number of 16S rRNA genes per genome for *Meiothermus* spp. were not known. Therefore, the calculations for qPCR based on arbitrary value for genome size (2.5 Mb)

assuming it contained 2 copies of the 16S rRNA gene. The accurate size of the genome of *Deinococcus geothermalis*, used by us as a reference DNA in qPCR with universal bacterial primers pE and pF', was not yet published either. I used for this the arbitrary values 3.2 Mb and 2 16S rRNA genes/genome. Since then, the genome sequences of *D. geothermalis* DSM11300^T, *M. ruber* DSM1279^T and *M. silvanus* DSM9946^T became publicly available (http://img.jgi.doe.gov, accessed January 2011) and revealed their genome sizes as 3.25, 3.10 and 3.72 Mb, respectively. The genome of *D. geothermalis* type strain contains four 16S rRNA genes whereas the sequenced *Meiothermus* species contain two 16S rRNA genes/genome.

Standard curve for the qPCR quantification of *Meiothermus* DNA was prepared with *M. silvanus* DSM9946^T (Figure 1 in Paper I). Based on the assumptions cited above, it was calculated that 1 ng of the purified DNA would contain 720 000 16S rRNA gene copies. Taking into account the presently known properties of the *M. silvanus* DSM9946^T sequenced genome, 1 ng of this DNA will contain 490 000 16S rRNA genes. This means that the correct 16S rRNA gene numbers of *M. silvanus* are one third lower than those presented in Paper I.

The standard curve for universal bacterial primers pE and pF' was drawn using results obtained with *D. geothermalis* E50051 DNA. At the time of the writing of the Paper I, it was assumed that 1 ng of *D. geothermalis* DNA contained 580 000 16S rRNA genes. The correct value for the type strain *D. geothermalis* DSM11300^T is 1 100 000 16S rRNA gene copies/ng, which is ~50% more than what we assumed for the strain *D. geothemalis* E50051. So, the numbers of Eubacterial 16S rRNA genes presented in Paper I may be doubled to achieve the closest estimate available today. Taking these uncertainties into account the current best estimate of the proportion of *Meiothermus* spp. in paper machine samples is lower than calculated in Paper I, but the change is not notable. For example, in Paper I it was concluded that in more than 10% of the biofilm samples *Meiothermus* spp. comprised >30% of the total bacterial 16S rRNA genes. The sequence information now available indicates that a better estimate may have been >10%. This new knowledge does not change the conclusions made in Paper I: *Meiothermus* spp. are major biofoulers in paper machines.

4.1.2 *Pseudoxanthomonas taiwanensis* is common throughout the paper making process

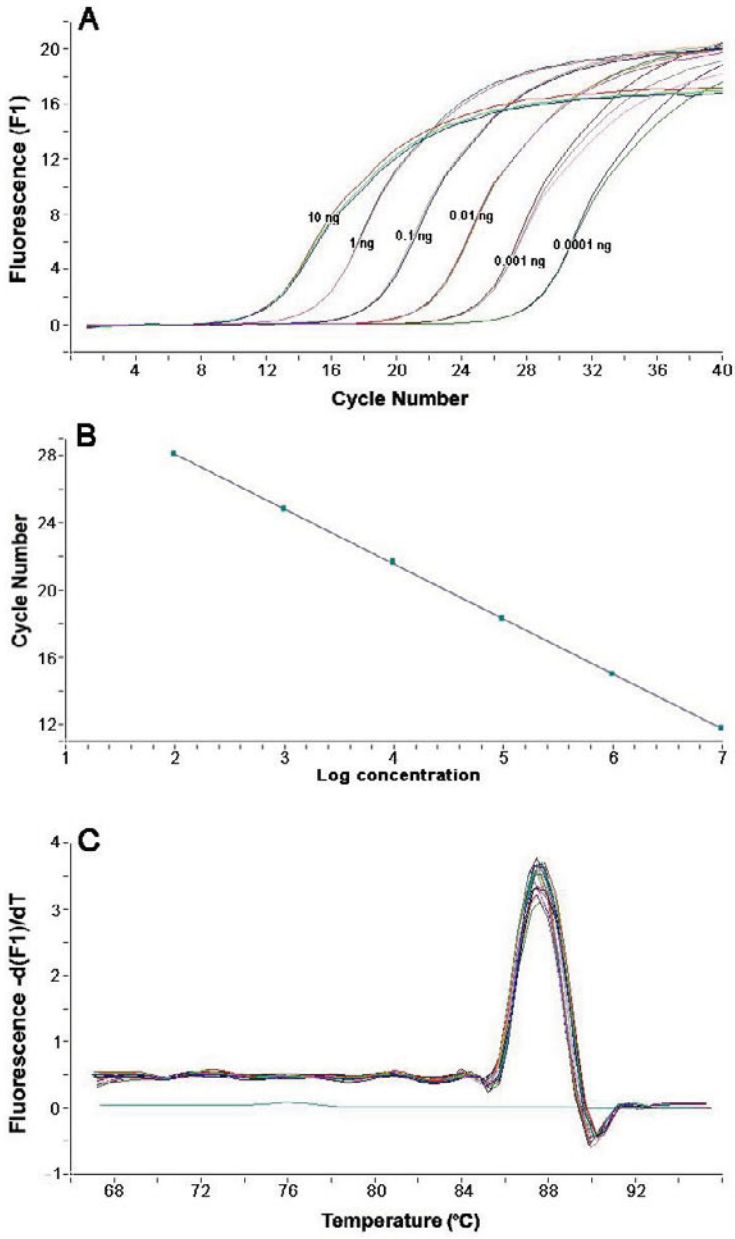


Figure 2. Quantitative PCR method for detection and quantification of *Pseudoxanthomonas taiwanensis*.
Panel A: Quantification of DNA from *Psx. taiwanensis* strain JN11003. Six different DNA concentrations were used, three replicate reactions each.
Panel B: Standard curve drawn from quantification shown on panel A.
Panel C: Melting curves of the amplification products of *Psx. taiwanensis* specific qPCR. DNA of *Psx. taiwanensis* strain JN11003 was used as the template.

Quantitative PCR method for *Pseudoxanthomonas taiwanensis* was developed (Figure 2). The figure shows that replicate reactions were quantified equally (Panel A) and quantification was log linear from 0.0001 to 10 ng DNA/reaction (78 – 7 800 000 16S rRNA gene copies/reaction (assuming genome size as 3.5Mb and three 16S rRNA gene copies per genome)) (Figure 2, Panel B). The melting temperature of the obtained PCR products was 88.0 ± 0.5°C (Figure 2, Panel C). Specificity of the primers was tested using DNA from nine non-target paper machine isolates: *Pseudoxanthomonas* sp. JN41003, *Pseudoxanthomonas* sp. S2-bf-WWA-8, *Pseudoxanthomonas broegbernensis* DSM 12573, *Porphyrobacter cryptus* A-col-BFA6, *Thermomonas haemolytica* DSM 13605, *Thermomonas hydrothermalis* JN31003 (Table 9), *Deinococcus geothermalis* E50051, *Pseudomonas boreopolis* S2-s-PMWA-6 and *Meiothermus silvanus* B-R2A5-50.4 (Table 1 of Paper I) and four strains not connected to paper machines: *Escherichia coli* MT102 (Table 1 of Paper I), *Staphylococcus epidermidis* DSM 20044, *Stenotrophomonas maltophilia* DSM 50170 and *Xanthomonas campestris* DSM 3586. None of these non-target DNA samples resulted in PCR products with correct melting temperature nor interfered with the quantification of *P. taiwanensis* J-M DNA when present in concentrations 100 times higher than the target DNA in the template mixture.

Figure 3 shows that *Pseudoxanthomonas taiwanensis* was found from all sample types examined, excluding the raw materials. *Psx. taiwanensis* DNA was also found in almost all machines studied. *Psx. taiwanensis* was reported from four machines in the study by Suihko *et al.* (2004). In many process water samples *Psx. taiwanensis* was the most numerous bacterium, sometimes comprising almost half of the bacterial load. Similar result was obtained earlier by Desjardins and Beaulieu (2003). They found that 62% of the isolates from the pulp slurries collected from the headbox of a Canadian paper mill represented genus *Pseudoxanthomonas* (Desjardins & Beaulieu 2003). *Psx. taiwanensis* thus is widespread in paper machines.

It has been noticed that *Psx. taiwanensis* can generate large aggregates with wood extractives in paper industry process waters (Leino *et. al*. 2011). This is an example of mechanisms how *Psx. taiwanensis* can be deleterious for the machine runnability since large aggregates can cause process failure more easily than small ones.

I found highest amounts of *Psx. taiwanensis* per gram of sample in biofilms of the wire section, headbox and disc filter. Even so, the proportion of *Psx. taiwanensis* in these samples remained low because the total bacterial numbers were also very high. This means that *Psx.*

taiwanensis was usually not the main biofilm former in these areas. The proportion of *Pseudoxanthomonas* was much smaller in wet-end biofilm than in pulps also in the study of Desjardins and Beaulieu (2003). They reported that 7 % of the isolates from slime represented the genus *Pseudoxanthomonas.*

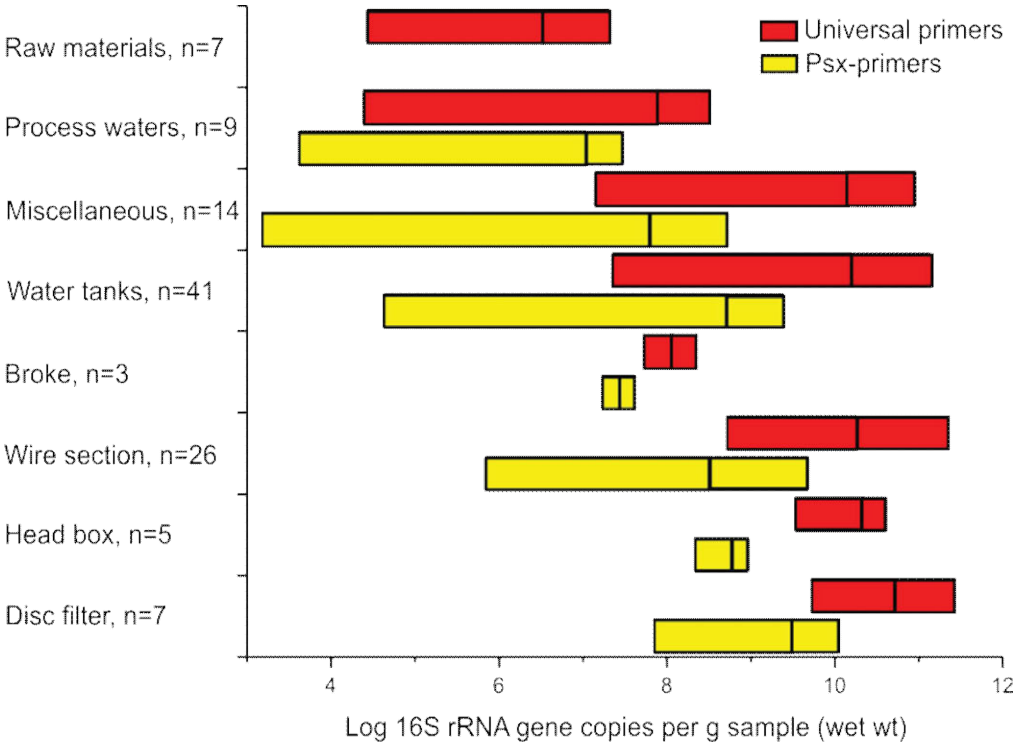


Figure 3. Bacterial and *Pseudoxanthomonas taiwanensis* 16S rRNA gene copy numbers in paper machines.
The samples were collected from different sites of more than 20 machines. The DNA was extracted and numbers of *Psx. taiwanensis* and total bacterial 16S rRNA genes were measured using qPCR. The floating bars indicate the highest and the lowest values per gram of sample (wet weight). The vertical lines inside the bars indicate the median of the values. No *Psx. taiwanensis* DNA was found in the raw materials (detection limit 10^2 – 10^4 16S rRNA genes per g of sample wet weight). n = number of samples from each location.

4.1.3 Bacteria found in end product defects

In Paper I I showed that DNA extraction followed by qPCR was useful for assessing possible causes of end product defects. This was done by extracting DNA from a visible spot and from a clean area on the same paper, in the vicinity of the spot. Figure 4 shows four examples of paper with end product defects. Their DNA amounts and 16S rRNA gene copy numbers with different primers are shown in Table 11. The defect in panel A contained 5

times more bacterial and 2 times more *Psx. taiwanensis* 16S rRNA genes than clean areas of the same paper. The defect in panel B contained about same amount of bacterial 16S rRNA gene copies as the clean area of the same paper. The small spots in panel C contained over 1000 times more bacterial 16S rRNA genes than the clean paper in the vicinity of the spots. In spite of the high DNA content, *Psx. taiwanensis* or *Meiothermus* spp. were not found from this sample (the amount of both *Meiothermus* spp. and *Psx. taiwanensis* DNA was below detection limit $<10^5$ 16S rRNA gene copies per g of paper). The defect in panel D contained 500 times more bacterial and *Psx. taiwanensis* 16S rRNA genes than the clean area of the same paper. It also contained 10^5 *Meiothermus* 16S rRNA genes, in comparison to the clean area which contained none ($<10^4$).

Taken together, the slimes or precipitates which caused the defects in panels C and D harboured high amounts ($>10^{10}$ 16S rRNA gene copies per g of paper) of bacterial DNA and were most likely caused by bacteria. Instead, the defect in panel B contained only small amount of bacterial (3×10^6 16S rRNA gene copies) DNA and thus was likely caused by non-microbiological precipitate. The defect in panel A contained quite high amount of bacterial DNA (2×10^9 16S rRNA gene copies per g of paper), but also the clean area of the paper contained moderate amount of bacterial DNA (4×10^8 16S rRNA gene copies per g of paper). This may reflect that the precipitate contained some bacteria, but they were not the main component of the defect. I suggest that in cases when a spot contains more bacterial 16S rRNA gene copies than clean area of the same paper, bacteria have played a role in formation of the defect.

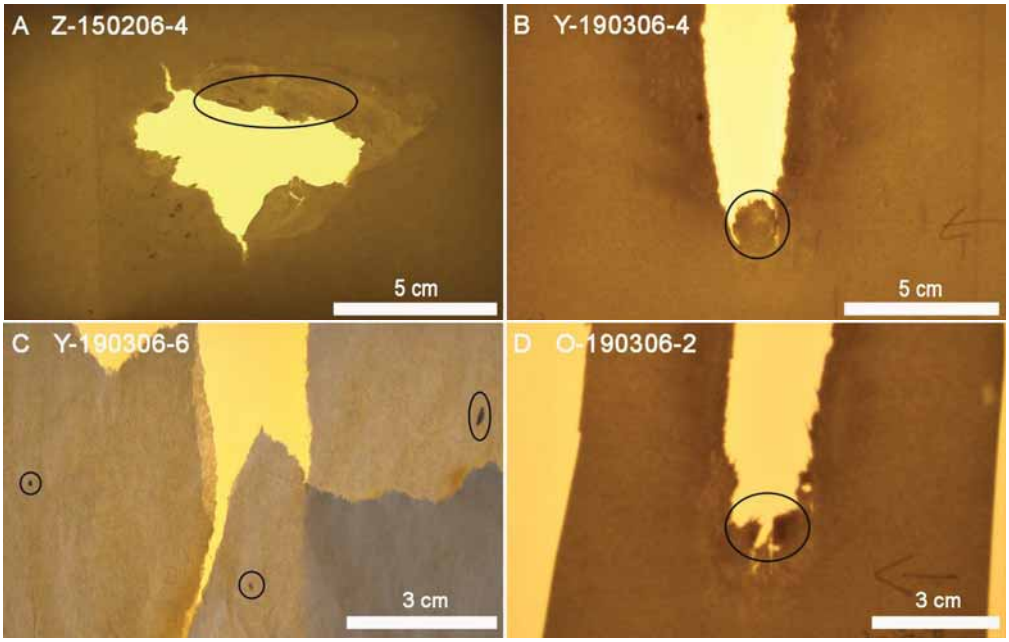


Figure 4. Examples of defects in paper machine end products.
Sample codes are indicated in each panel. Spot areas taken into DNA extraction are marked with circles.

Table 11 contains the results of 32 samples of defects from 8 paper machines. *Meiothermus* DNA was found in 18 defects but only in 3 clean papers. This shows that *Meiothermus* spp. were common in slimes causing end product defects but rare or in low numbers in raw materials and process waters, which constitute the clean paper. *Psx. taiwanensis* was found in all but four defects (88%) and also in 22 out of 26 analyzed clean papers (85%). The proportion of *Psx. taiwanensis* was high in many cases: In 11 defects and clean papers more than 10% of total bacterial 16S rRNA genes belonged to *Psx. taiwanensis*. The high prevalence of *Psx. taiwanensis* in clean papers is explained by the high prevalence of this bacterium in process waters (Figure 3). For example, in the process waters of machine R the proportion of *Psx. taiwanensis* 16S rRNA genes was 3% - 40% from total bacterial 16S rRNA gene copies. Both *Psx. taiwanensis* and *Meiothermus* spp. were found in majority of the studied machines (6/8 and 5/6, respectively), although the number of samples per machine was low (only 1-2 samples from six of the eight machines studied). This again illustrates the high prevalence of these bacteria in paper machines.

Table 11. 16S rRNA gene copy numbers in paper machine end products.

Sample	Amount of DNA (ng/g paper or board)		16S rRNA gene copy number g^{-1} paper or board					
			Universal bacterial primers pE and pF'[1]		*Pseudoxanthomonas* primers[2]		*Meiothermus* primers[3]	
	Spot	Clean	Spot	Clean	Spot	Clean	Spot	Clean
R-120905-2	10000	740	1×10^{10}	9×10^{8}	6×10^{8}	1×10^{8}	**3×10^{6}**	**6×10^{5}**
R-120905-4	1000	580	1×10^{9}	5×10^{8}	2×10^{8}	8×10^{7}	**5×10^{5}**	**4×10^{5}**
R-120905-6	7000	500	2×10^{9}	1×10^{8}	2×10^{8}	4×10^{7}	$<2\times10^{5}$	$<5\times10^{5}$
R-140905-2	2000	870	9×10^{8}	4×10^{8}	2×10^{8}	2×10^{8}	**6×10^{6}**	$<1\times10^{5}$
R-140905-4	8000	1000	7×10^{9}	6×10^{8}	2×10^{9}	3×10^{8}	**1×10^{6}**	**4×10^{5}**
R-140905-6	4000	930	3×10^{9}	4×10^{8}	2×10^{8}	9×10^{8}	**8×10^{8}**	**1×10^{5}**
R-140905-8	3000	950	2×10^{9}	4×10^{8}	2×10^{8}	2×10^{8}	**1×10^{5}**	$<1\times10^{5}$
R-140905-10	4000	890	6×10^{8}	3×10^{8}	8×10^{7}	7×10^{7}	**2×10^{6}**	$<3\times10^{5}$
R-140905-12	5000	820	5×10^{9}	6×10^{8}	2×10^{9}	1×10^{8}	**3×10^{6}**	$<1\times10^{5}$
R-151105-2	40000	500	3×10^{10}	3×10^{8}	3×10^{9}	2×10^{7}	**4×10^{6}**	$<2\times10^{5}$
R-151105-4	1000	600	9×10^{8}	3×10^{8}	8×10^{7}	5×10^{7}	**4×10^{5}**	$<2\times10^{5}$
R-151105-6	1000	600	1×10^{9}	3×10^{8}	1×10^{8}	4×10^{7}	**1×10^{6}**	$<1\times10^{5}$
R-151105-8	1000	500	8×10^{8}	4×10^{8}	6×10^{7}	2×10^{7}	**4×10^{5}**	$<2\times10^{5}$
R-151105-10	10000	300	6×10^{9}	3×10^{8}	8×10^{7}	2×10^{7}	**2×10^{6}**	$<1\times10^{5}$
R-151105-12	2000	600	2×10^{9}	5×10^{8}	3×10^{8}	4×10^{7}	**2×10^{5}**	$<1\times10^{5}$
R-151105-14	900	500	6×10^{8}	4×10^{8}	3×10^{7}	3×10^{7}	$<2\times10^{5}$	$<2\times10^{5}$
H-050805-2	7000	400	8×10^{9}	1×10^{8}	2×10^{7}	3×10^{6}	**4×10^{6}**	$<1\times10^{5}$
Z-150206-2	800	500	2×10^{8}	1×10^{8}	4×10^{6}	2×10^{6}		
Z-150206-4	3000	700	2×10^{9}	4×10^{8}	2×10^{5}	9×10^{4}		
W-200206-2	5000	800	4×10^{7}	2×10^{7}	1×10^{6}	5×10^{5}		
W-200206-8	1000	300	8×10^{8}	2×10^{8}	3×10^{5}	9×10^{3}		
Y-190306-2	10000	200	1×10^{10}	2×10^{7}	2×10^{9}	3×10^{6}	**1×10^{5}**	$<1\times10^{4}$
Y-190306-4	200	100	3×10^{6}	2×10^{6}	7×10^{4}	$<2\times10^{4}$	$<9\times10^{3}$	$<8\times10^{3}$
O-190306-6	70000	300	3×10^{10}	1×10^{7}	$<2\times10^{5}$	$<3\times10^{4}$	$<1\times10^{5}$	$<1\times10^{4}$
N-190306-8	30000	1000	2×10^{10}	1×10^{7}	$<2\times10^{4}$	$<4\times10^{4}$	$<1\times10^{4}$	$<2\times10^{4}$
N-190306-10	30000	400	2×10^{10}	2×10^{7}	$<9\times10^{3}$	$<3\times10^{4}$	**8×10^{4}**	$<2\times10^{4}$
SS-131005-1[4]	80		2×10^{7}		5×10^{5}		$<8\times10^{4}$	
SS-131005-2[4]	880		2×10^{9}		1×10^{6}		**3×10^{5}**	
SS-131005-3[4]	8000		6×10^{9}		1×10^{6}		$<9\times10^{4}$	
SS-131005-4[4]	1000		2×10^{10}		3×10^{6}		$<1\times10^{5}$	
SS-131005-5[4]	60000		1×10^{11}		8×10^{6}		$<2\times10^{5}$	
SS-131005-6[4]	6000		7×10^{9}		$<2\times10^{5}$		$<1\times10^{5}$	

[1]*D.geothermalis* E50051 DNA was used to create the standard curve. Gene copy numbers in standard samples were calculated assuming genome size 3.25Mb and 16S rRNA gene copy number of 4/genome.
[2] *Psx. taiwanensis* JN11003 DNA was used to create the standard curve. Gene copy numbers in standard samples were calculated assuming genome size 3.5Mb and 16S rRNA gene copy number of 3/genome. Samples where the proportion of *Pseudoxanthomonas* 16S rRNA genes was more than 10% of the total number are underlined
[3]*M. silvanus* DSM9946^T DNA was used to create the standard curve. Gene copy numbers in standard samples were calculated assuming genome size 3.72Mb and 16S rRNA gene copy number of 2/genome. Positive samples are marked bold.
[4]No clean paper was available.
In addition, 5 samples were studied but DNA amounts were so low that PCR method was not used.

Nine of the spot samples (Table 11) contained over 10 times more DNA than a clean area of the same paper. Also, nine spots harboured over 10 times more *Meiothermus* 16S rRNA genes than clean paper. High amounts of *Psx. taiwanensis* DNA were also found from clean areas of the papers. Only four spots with a 10 fold excess of *Psx. taiwanensis* 16S rRNA genes were found as compared to clean areas of those papers. My results concerning end product defects were published in two articles not included in this thesis (Peltola *et al.* 2008, Haapala *et al.* 2010). In the paper by Haapala *et. al.* (2010) causes of end product defects and web breaks were screened for. It was found that over 60% of the inspected 388 web breaks originated from holes or defects and that a majority of these contained bacterial DNA. This result indicates microbial involvement in the formation of holes or defects. It was also noticed that coloured (usually brown) defects often contained high amounts of bacterial DNA, whereas grey defects usually contained high amount of calcium carbonate. Defects with no significant colour were often caused by fragments of dry fibre material (Haapala *et al.* 2010). The paper by Peltola *et al*. (2008) reported that in defect paper the median number of the 16S rRNA gene copies was tenfold compared to clean paper. In addition to *M. silvanus* and *Psx. taiwanensis*, *D. geothermalis* DNA was found in two of the defects. Taken together, these findings show that bacterial biofilms growing on machine surfaces can cause end product defects and process failure.

4.1.4 Comparison of different methods for quantification of paper machine bacteria

It is well known that not all bacteria will grow on any single laboratory medium and that the choice of growth medium affects on the bacterial species that grow from the sample. Table 12 shows plate counting results from a paper machine white water on two growth media, PCA and R2A. PCA is a rich medium whereas R2A contains less nutrients. On PCA, the result was only 20 cfu/ml. Instead, the plate count on R2A was above detection limit (10 000

cfu/ml). Most bacteria grown on R2A presented a single type of small orange colonies. This colony type was never visible on PCA plates, indicating that the major bacterial species of that machine did not grow on PCA at all. This dramatic example shows how the choice of culture medium affects the results obtained by viable counting. During an earlier sampling, orange pigmented strain J-21.2 (Table 13) was isolated from this same paper machine. According to 16S rRNA gene sequence analysis, this isolate was closest related to *Porphyrobacter cryptus*, a bacterial species originally isolated from hot springs and cultivable on oligotrophic media (Rainey *et al.* 2003). The effect of culture media on the growing bacterial population from paper industrial samples has been noticed also earlier. On R2A 50% of the isolates could not be identified based on 16S rRNA sequencing because no known relative was included in the database. On PCA the corresponding number was only 27% (Desjardins & Beaulieu 2003). Kolari *et al*. (2003) isolated primary-biofilm formers from paper machines and noticed that many of the isolates could not grow at all on rich culture medium TSA.

Table 12. Cultivation of paper machine white water on rich (PCA) and on oligotrophic culture media (R2A)

Sample	Bacterial growth at 45°C for 2 d (cfu/ml)	
	PCA	R2A
White water, machine J	20	>10000

The bacterial amounts in five samples from the paper machine R were quantified with four different methods (Figure 5): By viable counting on two media, PCA and R2A, and by two culture independent methods, quantitative PCR and ATP-measurement. The results show that in three samples containing most bacteria (white water, clear filtrate and broke), the culture methods yielded somewhat higher results than the culture independent ones. The reason why qPCR gave lower bacterial numbers than culturing methods might be loss of DNA during DNA extraction procedure. The two culture media used (PCA and R2A) gave similar results. With qPCR it was noticed that the most prevalent bacterium in the white water, clear filtrate and broke was *Pseudoxanthomonas taiwanensis* (Figure 3), comprising 30 % of the total bacterial load of each sample. This bacterium grows well on PCA plates.

ATP measurement is fast (results within minutes) and it can be carried out on-site at the paper mill. As shown in Figure 5, the results obtained with ATP measurement were in line with those obtained using the three other methods. Thus, ATP measurement appears useful for the monitoring the bacterial levels in process waters of paper industry. Drawback of ATP measurement is that it does not distinguish the origin of ATP, *i. e*. the harmful and non-

harmful bacteria give same result. Recently, Kiuru *et al.* (2010) also found ATP measurement useful in their study carried out at fine paper machines.

In conclusion, the conditions between paper machines vary and same methods are not applicable in all situations. For example, bentonite could not be analyzed with ATP measurement at all (Figure 5). When culture medium is selected for a microbiological survey of paper machines, R2A is a safer choice than rich media (such as PCA, TSA or nutrient agar) because they may exclude large proportion of bacteria, as demonstrated in Table 12. Rich culture media have been the standard choice for cultivation of paper mill bacteria, but lately R2A has been used in several studies (for example Kolari *et al.* 2003, Desjardins & Beaulieu 2003, Kanto Öqvist *et al.* 2008, Kurissery *et al.* 2010, Rasimus *et al.* 2010).

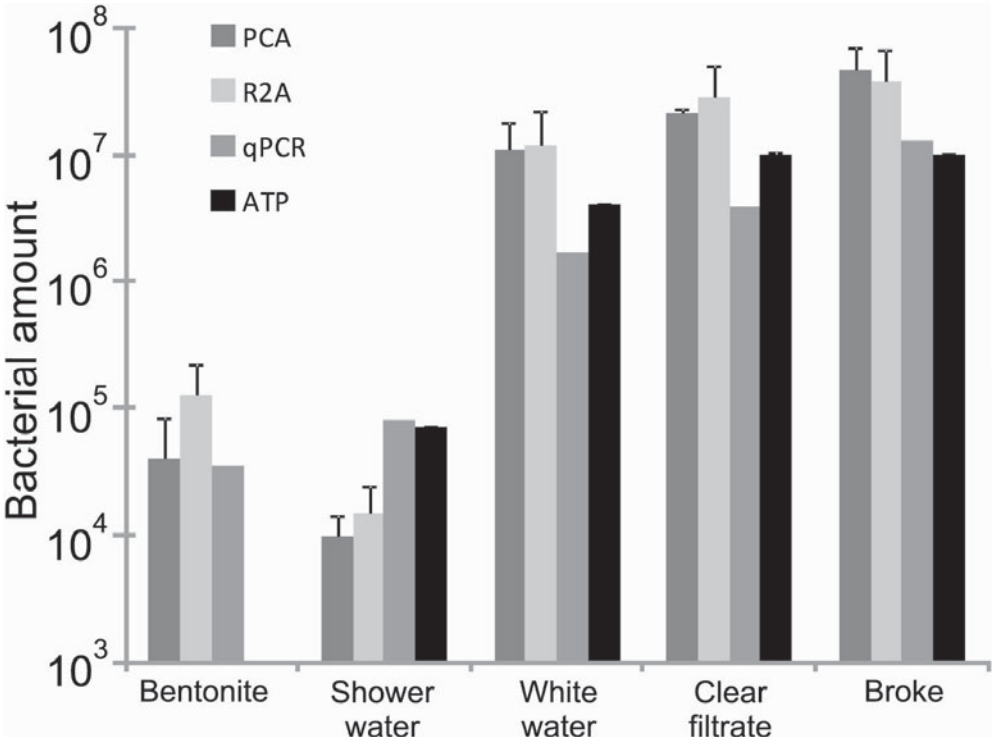


Figure 5. Bacterial amounts in paper machine samples using four different methods. Bacteria were plate counted on PCA and R2A, read after 2-3 d incubation at 45°C. Cultivations on R2A were averaged from the results obtained at four and those on PCA from two different laboratories. ATP was measured on-site directly after sampling, the other assays were done on the following day. Primers pE and pF' (Table 10) were used for the qPCR analysis. The numbers of 16S rRNA genes were converted into bacterial genome numbers by dividing with 4.1, which is the average 16S rRNA gene copy number in the sequenced bacterial genomes (rrndb.cme.msu.edu, accessed 23.1.2011, (Lee *et al.* 2009)). ATP readings were converted into bacterial cell numbers assuming ATP content as 2×10^{-18} mol/cell.

4.2 Bacteria isolated from paper machines during this thesis work

4.2.1 Primary-biofilm formers isolated from paper machines

Paper machines are open systems which continuously receive raw materials and water from external environment. Therefore, many bacterial species can be isolated from paper machines. Some of them can colonize paper machines or lead to process disturbances. In this study, I focused on isolating putative harmful bacteria, able of attaching surfaces and forming biofilm under paper machine conditions. The isolates, 35 from seven machines, isolated during this study are listed in Table 13 and in Table 6 of Paper I. Most of these originated from press felts and the others from biofilms at the wet-end. Biofilm forming abilities of the isolates were studied using the crystal violet assay (Kolari *et al.* 2003). The isolates were identified using 16S rRNA gene sequencing.

Six of the isolates (Table 13) were closely related to species of the genus *Pseudoxanthomonas* and another six closest to other genera of the family *Xanthomonadaceae*, namely *Stenotrophomonas*, *Silanimonas* and *Thermomonas*. These isolates originated from four different machines, both from the press felts and from wet-end biofilms. Seven of these isolates had highest 16S rRNA similarity to known species below 98 %, indicating that they may represent novel species. Before this study, *Psx. taiwanensis* was already reported from several paper machine samples (Table 1). With qPCR, I found that this species was common in many different paper machines and types of samples. It seems that *Psx. taiwanensis* and related bacteria are indeed major colonizers of paper machines.

Four isolates from press felts of three different machines were closely related to *Acinetobacter baumannii* and one isolate from a biofilm sample of yet another machine was related to *A. calcoaceticus*. Both of these species are opportunistic human pathogens and belong to hazard group 2 (Anonymous 2010). *A. baumannii* is a common cause of nosocomial infections (for example bacteriaemia, pneumonia, meningitis, infections of the urinary tract and of wounds). This species is often resistant to many antibiotics (Maragakis & Perl 2008). *Acinetobacter* strains were also earlier reported from samples of pulp and slime (Table 1).

Three *Bacillus* spp. (Table 13), growing in a filamentous pattern as viewed by microscope, were isolated from press felts of three different machines. To my knowledge, these are the first *Bacillus* isolates able to form biofilm in pure cultures in conditions mimicking paper

machine environment. For example, none of the isolates belonging to 7 *Bacillus* species, reported by Kolari *et al.* 2001, formed biofilm as pure cultures in paper machine water.

Five of the isolates (Table 13) were closest related to genus *Deinococcus*. One of them, strain K4.1^T, was described to represent a novel genus, named *Deinobacterium* (Paper III). Two of the isolates were assigned to the genus *Deinococcus* and were closely related to the type strain of *D. geothermalis*. This is a well known primary-biofilm former of paper machines (Kolari *et al.* 2001, Kolari *et al.* 2003, Peltola *et al.* 2008).

Meiothermus ruber was frequently isolated from machine L (Table 13 and Table 6 of Paper I). In contrast, no *Meiothermus* strains were isolated from another intensively studied machine K. With qPCR, *Meiothermus* spp. were found from many machines, often in large quantities and representing a high proportion of the total bacteria (Table 4 of Paper I).

Several of the bacterial species found from paper machines were first described from hot springs (*Deinococcus geothermalis, Meiothermus silvanus, Pseudoxanthomonas taiwanensis, Schlegelella aquatic, Tepidimonas* spp., *Thermomonas hydrothermalis*, Table 2). This thesis adds to the list *Deinococcus murrayi*, *Meiothermus ruber*, *Porphyrobacter cryptus* and *Silanimonas lenta* (Table 13) which also were first isolated from hot springs (Loginova *et al.* 1984, Ferreira *et al.* 1997, Rainey *et al.* 2003, Lee *et al.* 2005). It thus appears that most of the primary-biofilm formers at paper machines have their natural habitat in hot springs.

Some of the isolated bacterial species, namely *Bacillus panaciterrae, Deinococcus murrayi, Pannonibacter phragmitetus* and *Silanimonas lenta* (Table 13), have not previously been reported from paper machines. This, together with the fact that many of the biofilm forming isolates were not closely related to any currently known bacterial species, shows that there are still many novel biofilm formers waiting for discovery at the paper machines. Large proportion of unknown species of bacteria has also been noticed in other studies of paper machines, especially in those using culture independent methods (Lahtinen *et al.* 2006, Kanto Öqvist *et al.* 2008, Granhall *et al.* 2010).

Table 13. Biofilm forming bacteria isolated from paper machines during this thesis work.

Strain[1]	Biofilm production[2]	16S rRNA gene accession number	Closest relative based on 16S rRNA gene sequence[3]	Similarity (%)
H-s-1	3.9	FR774581	*Acinetobacter baumannii* MMC 16	99.9
L-s-27.1	3.3	FR774572	*A. baumannii* 29108 CMCC	100
L-s-29.1	3.5	FR774573	*A. baumannii* MMC 16	99.9
V-bf-R2-2	1.0	FR774568	*A. baumannii* LUH 5684	99.9
K-bf-R2A-8.2#	2.4***	FR774561	*A. calcoaceticus* NCCB 22016	99.2
V-bf-PMW-3	0.5	FR774586	*Bacillus pumilus* SB 3002	99.9
S-bf-PMW-5.1.1	1.0**	FR774585	*B. panaciterrae* rif200891	99.6[4]
L-bf-R2-30.3	1.8**	FR774584	*B. panaciterrae* rif200891	98.3
L-bf-R2A-15#	0.8**	FR774554	*Burkholderia multivorans* ATCC 17616	99.9
J-9.1#	0.6	FR774565	*Chelatococcus* sp. MW10	98.3
S-bf-PMW-5.3	1.6*		*Deinococcus cellulosilyticus* 5516J-15	96.6
L-bf-R2-30.2.1	4.1*	FR774583	*D. geothermalis* DSM 11300	100
K-bf-R2A-4.3.1#	4.3	FR774556	*D. geothermalis* DSM 11300	99.9
S-bf-PMW-8.5.1	1.3	FR774569	*D. murrayi* RSPS-7a	98.4[4]
K4.1T#	2.0	AM988777	*D. pimensis* KR-235	90.0
L-s-R2A-4C.1#	1.1	AM229087	*Meiothermus ruber* DSM 1279	99.2
L-s-R2A-4B.2.1#	2.1	AM229088	*M. ruber* DSM 1279	99.7
L-s-R2A-4B.2.3#	3.3	AM229089	*M. ruber* DSM 1279	99.7
L-s-PMW-4B.2.1#	1.6	AM229090	*M. ruber* DSM 1279	99.7
L-s-PMW-4B.2.2#	2.3	AM229091	*M. ruber* DSM 1279	99.7
L-s-PMW-4C.1#	3.2	AM229092	*M. ruber* DSM 1279	99.7
L-s-PMW-11.1#	4.5	AM229093	*M. ruber* DSM 1279	99.7
L-bf-PMW-16.2.2	1.3	AM229094	*M. ruber* DSM 1279	99.7
L-bf-PV-PMW-3B.1#	1.2	AM229095	*M. ruber* DSM 1279	99.2
L-s-R2A-3B.2#	3.8	AM229096	*M. ruber* DSM 1279	99.6
L-bf-PV-PMW-3B.2.3#	2.3	AM229097	*M. ruber* DSM 1279	99.2
L-bf-PMW-16.1.2.1	1.2	AM229098	*M. ruber* DSM 1279	99.2
L-s-R2A-4C.2#	4.5	AM229086	M. silvanus DSM 9946	99.9
L-s-29.2	3.6	FR774577	*Microbacterium* sp. cp-h5	99.2
S-s-1	1.5	FR774571	*Microbacterium* sp. cp-h5	99.3

J-21.2#	0.9	FR774566	*Porphyrobacter cryptus* ALC-2	99.7
L-s-R2A-19.4	2.4	FR774557	*Pannonibacter phragmitetus* LMG 5412	99.9
L-bf-R2A-1.1.1#	2.7**	FR774558	*Pseudoxanthomonas mexicana* AMX26B	96.8
L-s-28.1	3.1	FR774579	*Psx. mexicana* AMX26B	96.7
L-bf-PMW-31.1	0.4	FR774578	*Psx. taiwanensis* J-M	100
L-bf-R2A-13.2.3#	4.1	FR774559	*Psx. taiwanensis* J-M	99.9
S-p-2	3.1	FR774574	*Psx. suwonensis* 4M1	98.5
S-s-6.1	4.3	FR774580	*Psx. suwonensis* 4M1	98.5
L-bf-PMW-29.6	2.4	FR774564	*Rubellimicrobium thermophilum* C-lvk-R2A-2^T	99.8
L-bf-PV-PMW-3A.2.1#	1.1	FR774555	*R. thermophilum* E-R2A-8a	100
L-bf-PMW-28.2	2.9	FR774570	*Schlegelella aquatica* wcf1	99.3
P-bf-PMW-1	1.3	FR774567	*S. thermodepolymerans* SA1	98.5
L-bf-PMW-29.5	4.3	FR774582	*Silanimonas lenta* 25-4	99.6
L-bf-PMW-31.3	3.4	FR774575	*Stenotrophomonas* sp. AGL 1	96.8
S-bf-PMW-2	4.3	FR774576	*Stenotrophomonas* sp. AGL 1	97.5
L-s-28.2	1.4	FR774563	*Stenotrophomonas* sp. AGL 1	97.5
K-bf-R2A-11.3#	4.4	FR774562	*Thermomonas haemolytica* A50-7-3	97.5
L-bf-PMW-15.1#	4.6**	FR774560	*Xanthomonadaceae* TDMA-47	98.0

[1]Strains were isolated from used press felts or wet-end biofilms (marked #). First letter of the strain code indicates the machine from which the strain in question was isolated. So, the strains originated from 7 different machines.

[2] The strains were grown in three replicate wells of polystyrene microplates for 3 d in R2A broth or paper machine water (indicated with *) at 45°C, at 37°C (**) or at 28°C (***). After the incubation, the biofilms grown onto the surfaces of the wells were stained with crystal violet. Bound dye was solubilised with ethanol and quantified using microplate reader (A_{595}). The number in the table indicates the color intensity, which reflects the biofilm volume.

[3]According to FASTA (Pearson & Lipman 1988; run 17.9. – 6.10.2010)

[4]Sequence length less than 1000 base pairs

4.2.2 *Deinobacterium chartae*, a novel biofilm forming species isolated from a paper mill

Strain K4.1^T was isolated from a biofilm growing in the wire section of a paper mill producing folding boxboard. Polyphasic taxonomic study was carried out to characterize it and based on that study *Deinobacterium chartae* gen. nov., sp. nov. was described, strain K4.1^T being the type strain of this novel genus (Paper III).

Table 14. Main characteristics of *Deinobacterium chartae*

Cell morphology	Rod, 0.8-1.3 µm ×1.4-2.5 µm
Metabolism	Aerobic, chemo-organotrophic
Gram reaction	Negative
Colony colour on R2A plate	Pale pink
Optimal growth temperature	37 - 45°C
Growth pH	6 – 10.3
DNA G+C content	66.8%
Peptidoglycan type	A3β (L-Orn - Gly – Gly)
Menaquinone type	MK-8
Major polar lipid	Unknown aminophospholipid
Major fatty acids	15:0 iso and anteiso, 17:0 iso and anteiso
Radiation resistance	Equal to *D. radiodurans* DSM 20539^T, *i.e.* 10 kGy caused < 1 log viability loss
Oxidase and catalase reactions	Positive
Motility	No
Spores	No

Cells of the strain K4.1^T are rod shaped and they stain Gram-negatively (Figure 6, Panels A and B). The length of the cells was 1.4-2.5 µm (grown on TSA for 3 d at 45°C). Cells were much longer (3 - 8 µm) when grown in rich liquid medium (TSB) at 45°C for 1 d and filamentous (up to 40 µm long) after 2 d (Figure 6 panels C and D, respectively). *Deinobacterium chartae* is not the only bacterium with variable cell length. For example, species of the genus *Meiothermus* are known to form long filaments under some culture conditions (Nobre & da Costa 2001).

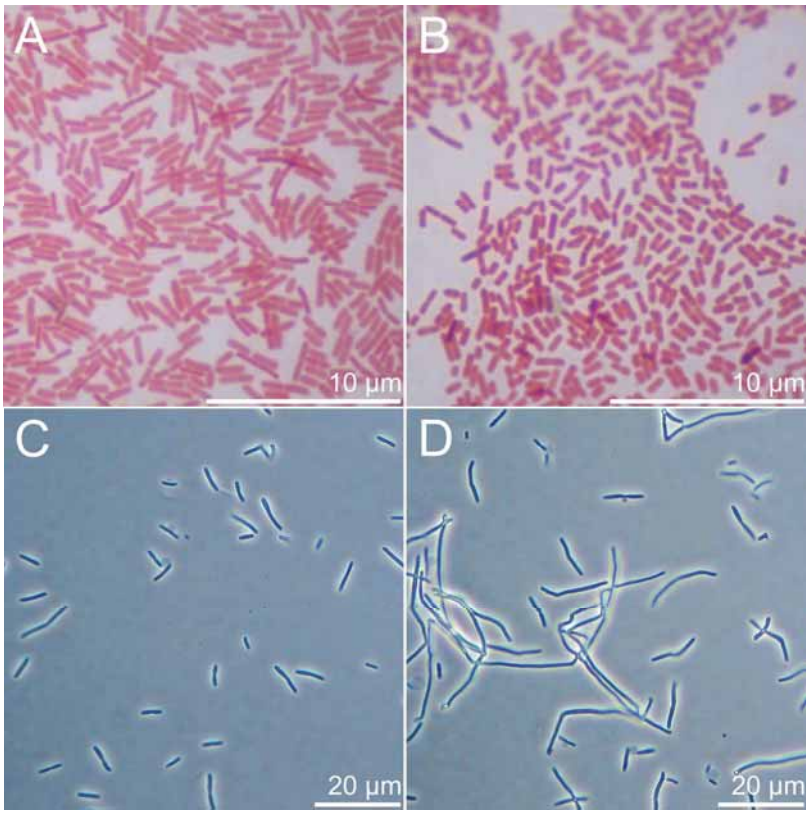


Figure 6. Microscopic images of *Deinobacterium chartae* strain K4.1^T.
A and B: Gram-stained cells grown on TSA plates for 1 d at 37°C (A) or at 45°C (B).
C and D: Phase contrast images of cells grown in TSB at 45°C for 1 d (C) or for 2 d (D).

According to the 16S rRNA gene sequence, the closest phylogenetic relatives of the strain K4.1^T were *Deinococcus pimensis* (similarity 90.0%), *D. peraridilitoris* (89.4%) and *D. maricopensis* (89.6%). In phylogenetic trees (Figure 7 and Supplementary figure S4 of Paper III) the strain K4.1^T formed a distinct branch at the base of the genus *Deinococcus*. The tree in the supplementary figure S4 of the Paper III was constructed using the maximum-likelihood algorithm and includes all type species of the genus *Deinococcus* known at the time of the submission of the article (June 2009). The tree in Figure 7 was created using weighted neighbor joining algorithm and it contains all 45 currently (November 2010) described *Deinococcus* species.

In addition to the distinct phylogenetic position, the results of polar lipid analysis strongly supported placing strain K4.1^T in its own genus. The predominant lipid of the strain K4.1^T was an aminophospholipid (APL1, Figure 2 of Paper III) whereas members of the genus *Deinococcus* have a phosphoglycolipid as the predominant lipid. Deinococci also contain other phosphoglycolipids, whereas no kind of phosphoglycolipid was detected in the strain K4.1^T. The major lipid APL1, together with the complete absence of phosphoglycolipids, distinguishes strain K4.1^T from all *Deinococcus* species examined for polar lipids.

I analyzed the whole cell fatty acid profile of the strain K4.1^T from biomass grown under three different conditions (Table 1 of Paper III). The main fatty acids of the strain K4.1^T were unsaturated and branched (15:0 iso, 15:0 anteiso, 17:0 iso and 17:0 anteiso). Strain K4.1^T differed from *Deinococcus* species by absence of the straight chain unsaturated and cis-monunsaturated fatty acids, which are the major components in most deinococci (Table 2 and Supplementary table S1 of Paper III). Strain K4.1^T also contained ~3 % of hydroxy fatty acids (17:0 iso 3OH and 17:0 2OH), which were absent or scarce in deinococci.

4.3 Future of the genus *Deinococcus* – Should it be divided into many genera?

In paper III we divided genus *Deinococcus* into 8 clades based on their whole cell fatty acid compositions (Table 2 of Paper III and Supplementary Table 1 of Paper III). This was done because the fatty acid compositions vary remarkably between the species of the genus *Deinococcus* and therefore it was not possible to distinguish *Deinobacterium chartae* or *Truepera radiovictrix* from the whole genus *Deinococcus* based on their fatty acid composition. Instead, *Deinobacterium* and *Truepera* are clearly separated from each of the Deinococcal clades. Thirteen of the 38 species of deinococci fitted to none of the clades.

Clades proposed in Paper III might serve as a starting point when genus *Deinococcus* will be divided into several more coherent genera than the present one. The clades (some recently described species were added into the clades) are marked with different colours in the phylogenetic tree (Figure 7), together with some morphological information (rod/coccus, staining Gram-positively/-negatively). It should be noted that in the phylogenetic trees only minority of the branches within the genus *Deinococcus* are supported by high (>70%) bootstrap values (Figure 7 and Supplementary Figure S4 of Paper III). Thus, species in the different branches of the trees may actually represent a same clade. The 16S rRNA gene sequence similarities between all current species of *Deinococcus* are presented in Table 15.

Clades III and VIII comprise seven coccus shaped species (*Deinococcus frigens*, *D. saxicola*, *D. marmoris*, *D. hopiensis*, *D. xibeiensis*, *D. wulumiqiensis* and the type species of the genus, *Deinococcus radiodurans*). Based on the fatty acid profiles, these two clades are close, the main difference being the higher amount of the *cis*-monounsaturated 16:1 and 18:1 fatty acids in the clade VIII. The 16S rRNA gene sequence similarities between these species are 89.2 – 100% (Table 15). They, or some of them, may form the core of the redefined genus *Deinococcus*.

Most distinct among the clades is number VI, comprising of *Deinococcus roseus*, *D. misasensis* and *D. cellulosilyticus*. Based on the 16S rRNA gene sequence similarities, these three species are close to each other (similarities 98.0 – 98.3%, Table 15) but distant to all other species of the genus *Deinococcus* (84.5-89.6%). *Deinococcus roseus*, *D. misasensis* and *D. cellulosilyticus* contain no or only minute amounts of monounsaturated fatty acids, which are the major fatty acids in most of the other species of deinococci. Instead, they have high amounts of iso- and anteiso-branched saturated fatty acids, and actually their fatty acid compositions are more reminiscent to those of *Deinobacterium* and of *Truepera* than of *Deinococcus* (Table 2 of Paper III and Supplementary Table 1 of Paper III). The reason for this might be adaptation to warm aqueous environment: *Deinobacterium chartae* was isolated from a paper machine and *T. radiovictrix*, *D. roseus* and *D. misasensis* from hot springs (Albuquerque *et al.* 2005, Asker *et al.* 2008). To my opinion, it is safe to say that this clade should be separated from the genus *Deinococcus*.

Clades I and VII (*Deinococcus indicus, D. ficus, D. grandis, D. deserti, D. navajoensis, D. hohokamensis, D. caeni, D. aquaticus* and *D. gobiensis*) might form the core for a third genus. Whole cell fatty acid compositions of these clades are similar, the largest difference being that the species of clade VII have a higher proportion of fatty acids with even number of carbons than those of the clade I. Most of these species are rod shaped and stain Gram-negatively. Whole cell fatty acid compositions of two other rod shaped species, *D. aquatilis* and *D. depolymerans*, are close to these clades, although the full fatty acid profile of *D. depolymerans* is not yet available (Asker *et al.* 2010). The 16S rRNA gene sequence similarities between these species are 91.8 – 98.3%.

Clade V comprises *Deinococcus altitudinis*, *D. claudionis* and *D. radiomollis*. Phylogenetically they are relatively close to each other (similarities 96.9 – 97.5%, Table 15) and also similar to *D. aquiradiocola* (95.8 - 96.7%), but distant to all other species of the present genus *Deinococcus* (similarities ≤92.7%). They are all rod shaped, stain Gram-positively and form their own branch in the phylogenetic trees (Figure 7 and Supplementary Figure S4 of Paper III) within the genus *Deinococcus*. The whole cell fatty acid composition of *D. aquiradiocola* differs from those of clade V, but it may be because it was grown for the fatty acid analysis at higher temperature and on a medium different from that used for the other three other species (Supplementary Table 1 of Paper III). The polar lipids of these species include aminophospholipids (Callegan *et al.* 2008, Asker *et al.* 2009), which are rare among the other deinococci. In addition, *D. altitudinis*, *D. claudionis* and *D. radiomollis* were reported to be only moderately radiation resistant (Callegan *et al.* 2008). *D.*

aquiradiocola was reported to be radiation resistant but only two doses, 2.3 kGy and 16 kGy, were tested (Asker *et al.* 2009) and therefore it is difficult to say whether this species is more irradiation resistant than are the species of the clade V.

A further new genus might originate from the clade IV, holding species *Deinococcus maricopensis*, *D. yavapaiensis* and *D. pimensis*, of which all are rod shaped and stain Gram-positively. These species have no close phylogenetic relative. 16S rRNA gene sequence similarities within these species are only 89.6 – 91.2, but most of the other species are even more distant. Based on the 16S rRNA gene sequence similarities, also *D. peraridilitoris* and *D. papagonensis* might be included into this clade.

In order to split the genus *Deinoccus* into novel genera, a more comprehensive polyphasic characterization of the type strains of the genus *Deinococcus* should be carried out. As the 16S rRNA sequence similarity values between many of the species are low, analysis of other genes would be useful. The genomes of *D. radiodurans* R1 (White *et al.* 1999), *D. geothermalis* DSM11300 (Makarova *et al.* 2007) and *D. deserti* VCD115 (de Groot *et al.* 2009) are currently available. When more genomes are sequenced, comparison of the genomes will be possible and may help reclassification of the genus. In addition, whole cell fatty acids of all deinococci should be analysed under similar cultivation conditions to allow proper comparison of the fatty acid compositions between the species. Polar lipids have not been analyzed from all species of *Deinococcus*. This information would be a very interesting tool for taxonomic analysis of the genus *Deinococcus*.

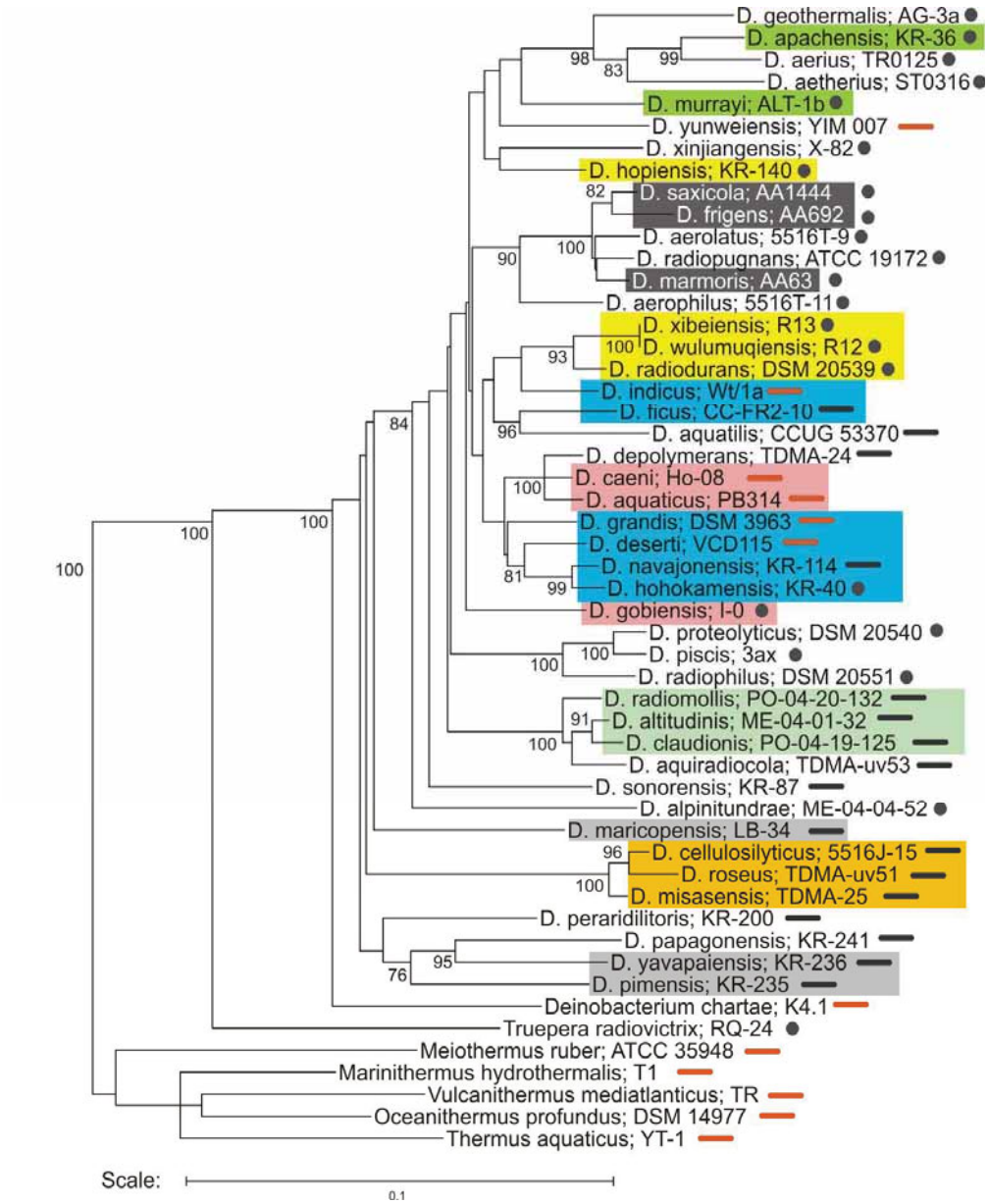


Figure 7. A phylogenetic tree constructed from the 16S rRNA gene sequences of *Deinobacterium chartae* K4.1^T and 51 species from the phylum *Deinococcus-Thermus.* The aligned sequences were retrieved from the Ribosomal Database project II (RDP) (Cole *et al.* 2009). The phylogenetic tree and the bootstrap values (100 resamplings) were constructed using weighted neighbour joining method (Bruno *et al.* 2000) and the Tree Builder of the RDP (http://rdp.cme.msu.edu). Bootstrap values over 70% are given next to the corresponding branches. Cell morphology is indicated with symbol (rod ▬, coccus ●) and Gram-negatively staining species are marked red. Clades of the genus *Deinococcus* (Paper III) are painted with colours. *Chloroflexus aggregans* was used as an outgroup.

Table 15. 16S rRNA gene sequence similarities between *Deinobacterium* and *Deinococcus* species.

	K4.1	Dpim	Dper	Dmar	Dcae	Dhoh	Dgeo	Dros	Dpis	Dpro	Dnav	Dgra	Dfic	Dgob	Dmis	Daqc	Dmur	Dson	Dpap	Dind	Dalt	Dyav	Dalp
K4.1		90.0	89.6	89.4	89.1	89.0	88.9	88.9	88.9	88.7	88.5	88.5	88.5	88.4	88.4	88.4	88.4	88.3	88.2	88.1	88.1	88.0	87.9
Dpim	90.0		90.9	90.8	89.0	88.5	89.8	86.7	88.7	88.4	88.1	88.4	88.7	89.2	87.4	88.3	88.2	89.7	91.4	87.9	88.0	91.2	88.1
Dper	89.6	90.9		90.8	89.8	89.3	89.4	87.7	90.5	90.3	89.0	89.2	88.7	90.9	88.7	89.7	89.5	90.8	90.7	89.5	89.0	90.1	89.4
Dmar	89.4	90.8	90.8		89.6	90.0	87.9	88.5	89.2	89.6	89.4	90.0	90.6	89.8	88.6	89.3	88.2	90.8	89.3	89.2	90.1	89.6	90.0
Dcae	89.1	89.0	89.8	89.6		95.0	90.5	88.6	91.6	91.3	94.4	96.8	93.2	93.5	88.9	98.3	92.4	91.7	88.1	96.6	91.7	88.4	89.9
Dhoh	89.0	88.5	89.3	90.0	95.0		89.8	87.3	91.6	91.5	98.4	95.2	93.4	93.5	87.7	94.6	92.1	91.3	87.4	94.0	90.6	87.7	89.9
Dgeo	88.9	89.8	89.4	87.9	90.5	89.8		86.7	89.9	90.1	89.0	89.7	89.9	90.8	87.2	90.3	91.9	89.5	88.2	90.6	88.5	88.3	88.7
Dros	88.9	87.4	88.5	89.4	89.6	88.3	87.5		87.4	87.7	87.8	88.2	87.9	87.0	98.2	89.4	87.7	87.8	86.4	88.7	87.3	87.1	86.7
Dpis	88.9	88.7	90.5	89.2	91.6	91.6	89.8	86.4		98.2	90.8	91.6	90.3	91.9	86.8	91.7	89.3	90.0	88.3	91.2	90.3	87.0	89.0
Dpro	88.7	88.4	90.3	89.6	91.3	91.5	90.1	86.8	98.2		90.6	91.5	90.6	92.4	86.7	91.6	89.9	90.1	88.3	91.5	90.5	87.1	88.9
Dnav	88.5	88.1	89.0	89.4	94.4	98.4	89.0	86.8	90.8	90.6		94.8	94.1	93.3	87.3	94.3	91.4	90.7	87.1	93.7	91.0	87.5	89.7
Dgra	88.5	88.4	89.2	90.0	96.8	95.2	89.7	87.2	91.6	91.5	94.8		94.9	93.4	87.4	96.5	91.8	91.2	87.6	95.1	91.1	88.3	89.6
Dfic	88.5	88.7	88.6	90.6	93.2	93.4	89.9	87.0	90.2	90.5	94.1	94.9		92.3	87.4	93.4	91.3	91.0	87.3	93.2	91.7	87.5	89.0
Dgob	88.4	89.2	90.9	89.8	93.5	93.5	90.8	86.2	91.9	92.4	93.3	93.4	92.3		86.3	94.0	91.1	91.5	89.1	93.2	90.9	88.8	90.2
Dmis	88.4	87.6	89.1	89.0	89.4	88.1	87.5	98.2	87.4	87.3	87.9	87.8	88.0	86.9		89.4	87.2	87.6	87.0	88.6	87.7	87.2	87.0
Daqc	88.4	88.3	89.7	89.3	98.3	94.6	90.3	88.4	91.7	91.6	94.3	96.5	93.4	94.0	88.9		91.9	92.3	87.9	96.6	92.6	88.4	90.1
Dmur	88.4	88.2	89.5	88.2	92.4	92.1	91.9	86.8	89.3	89.9	91.4	91.8	91.3	91.1	86.6	91.9		91.1	87.6	92.0	90.1	89.2	89.1
Dson	88.3	89.7	90.8	90.8	91.7	91.3	89.5	86.9	90.0	90.1	90.7	91.2	91.0	91.5	87.2	92.3	91.1		88.8	91.4	91.2	90.4	89.2
Dpap	88.2	91.4	90.7	89.3	88.1	87.4	88.2	85.4	88.3	88.3	87.1	87.6	87.3	89.1	86.5	87.9	87.6	88.8		87.7	87.4	92.4	87.2
Dind	88.1	87.9	89.5	89.2	96.6	94.0	90.6	87.7	91.2	91.5	93.7	95.1	93.2	93.2	88.1	96.6	92.0	91.4	87.7		91.3	87.6	89.6
Dalt	88.1	88.1	89.0	90.1	91.7	90.6	88.5	86.3	90.3	90.5	91.0	91.1	91.7	90.9	87.1	92.6	90.2	91.2	87.4	91.3		87.3	89.2
Dyav	88.0	91.2	90.1	89.6	88.4	87.7	88.3	86.2	87.0	87.1	87.5	88.3	87.5	88.8	86.8	88.4	89.2	90.4	92.4	87.6	87.3		86.0
Dalp	87.9	88.1	89.4	90.0	89.8	89.9	88.7	85.7	88.9	88.9	89.7	89.6	88.9	90.2	86.4	90.1	89.1	89.2	87.2	89.6	89.2	86.0	
Drml	87.8	88.1	89.2	90.9	91.9	91.2	88.7	85.6	89.7	90.1	91.4	91.4	91.8	92.0	86.7	92.7	90.6	92.2	87.7	91.9	96.9	88.1	88.8
Dcel	87.8	87.8	89.2	88.3	89.7	87.7	87.5	98.0	87.8	88.1	87.5	87.7	87.6	87.1	98.3	89.7	87.3	87.6	86.4	88.7	87.4	86.6	86.8
Drph	87.8	87.4	89.5	89.1	91.5	91.2	89.8	86.9	95.6	95.1	90.5	91.1	90.3	91.2	86.9	91.6	90.4	89.4	87.2	91.9	90.9	86.9	88.0
Dyun	87.8	87.9	87.7	88.9	91.9	91.2	90.3	85.9	89.1	89.0	91.4	91.8	92.2	91.7	86.0	92.3	90.6	91.3	87.7	92.0	89.0	87.5	87.8
Ddes	87.7	89.1	88.8	90.6	94.8	96.1	89.3	87.2	92.3	91.8	97.1	95.0	94.0	93.6	87.7	95.0	91.2	91.3	88.4	93.2	92.0	88.1	89.5
Dapa	87.6	88.2	88.4	87.2	89.7	90.0	93.4	85.1	89.0	89.2	88.9	89.4	88.7	89.4	85.4	89.5	91.8	89.0	87.9	89.5	88.4	87.7	89.0
Drdr	87.6	87.6	89.4	89.9	93.5	93.1	90.0	86.3	90.9	91.1	93.1	93.6	93.3	92.9	87.0	93.7	91.0	90.9	87.3	96.3	90.8	87.6	89.2
Daet	87.6	88.7	86.9	87.7	90.1	89.1	91.6	84.8	87.8	87.9	88.4	89.7	90.0	89.5	84.5	90.1	89.4	89.4	87.8	89.9	87.6	87.9	87.5
Dcla	87.5	88.2	89.1	89.7	91.4	90.9	88.2	85.2	90.4	90.6	91.3	90.9	91.5	92.0	86.2	92.2	89.7	91.9	87.6	91.0	97.5	87.6	88.5
Dmrm	87.4	86.3	88.6	88.9	93.6	91.9	88.7	86.1	90.1	90.3	91.8	92.7	91.4	91.5	85.8	93.2	91.8	89.9	86.2	93.0	90.2	87.2	88.3
Dwul	87.4	87.3	89.8	89.6	92.7	92.6	89.9	86.0	92.4	92.4	92.4	93.0	93.3	92.3	86.8	92.5	90.7	90.3	86.5	94.9	90.8	86.4	88.9
Darl	87.3	86.0	88.0	88.8	93.3	92.9	88.9	87.3	89.5	89.5	92.9	92.9	92.7	91.7	86.8	93.3	92.1	89.7	85.6	91.7	91.0	86.9	88.2
Darp	87.3	86.4	87.9	88.9	93.2	93.9	89.9	86.6	90.5	90.0	93.1	93.4	92.6	92.7	86.4	93.0	92.2	90.1	86.1	92.6	89.8	87.0	89.9
Dars	87.2	88.9	88.7	88.2	89.2	88.9	92.9	85.1	87.9	88.3	88.1	88.9	88.3	89.9	85.4	89.4	91.1	88.8	88.0	89.5	87.4	88.6	88.4
Dxib	87.2	87.3	89.7	89.6	92.5	92.5	89.7	86.4	92.2	92.3	92.4	92.9	93.2	92.1	86.8	92.3	90.6	90.2	86.4	94.7	90.7	86.3	88.9
Daqr	87.2	87.8	89.1	88.9	91.3	90.5	88.2	86.9	89.9	90.1	90.6	90.2	90.8	90.8	86.7	91.8	89.9	90.8	87.4	90.7	96.7	87.3	88.4
Drpg	87.1	86.5	87.9	88.4	92.7	91.9	88.7	85.4	90.2	91.0	91.8	93.5	91.9	91.6	85.4	92.8	91.8	90.4	87.0	92.1	90.5	87.3	88.2
Dfri	87.1	85.8	87.9	87.9	92.4	91.9	89.0	84.5	89.9	90.2	91.8	93.0	90.7	91.5	84.7	92.3	90.8	88.8	85.8	91.3	89.7	86.5	88.6
Daql	87.1	86.7	89.1	88.1	93.3	93.3	89.1	87.7	89.7	90.1	94.0	93.3	94.3	92.2	88.3	93.3	91.1	90.5	85.8	93.1	91.3	86.4	88.3
Ddep	87.1	87.5	88.2	88.6	97.0	94.0	88.9	89.2	90.2	90.6	93.2	94.9	91.8	92.8	89.0	96.6	90.9	90.5	87.0	95.6	90.9	87.4	88.7
Dsax	87.0	86.0	88.2	88.1	93.4	91.8	88.4	85.6	90.5	90.7	91.6	92.7	90.4	92.0	85.6	93.1	91.3	89.5	86.1	92.1	90.1	86.9	89.1
Dxin	87.0	88.0	89.2	88.1	92.6	90.9	88.9	86.4	90.2	90.6	91.6	91.4	93.1	92.2	86.0	93.0	89.9	89.3	87.2	92.5	90.5	87.5	89.5
Dhop	86.7	88.1	89.7	89.3	92.8	93.4	89.3	86.5	90.7	91.1	93.3	93.1	92.3	92.2	87.0	93.2	92.3	91.1	87.4	93.5	91.8	87.1	89.9

16S rRNA gene sequence similarities were calculated using Similarity Table analysis provided at www.eztaxon.org (Chun *et al.* 2007). Colours indicate the similarity of the sequences: The redder the colour, the closer the sequences are to each other.Dars, *D.aerius;* Darl, *D.aerolatus*; Darp, *D.aerophilus*; Dalp *D.alpitundrae;* Dalt, *D.altitudinis;* Dapa, *D.apachensis;* Daqr, *D. aquaradiocola;* Daqc, *D.aquaticus;* Daql, *D.aquatilis;* Dcae, *D.caeni;* Dcel, *D.cellulosilyticus;* Dcla, *D.claudionis;* Ddep, *D.depolymerans;* Ddes, *D.deserti;*

	Drml	Dcel	Drph	Dyun	Ddes	Dapa	Drdr	Daet	Dcla	Dmrm	Dwul	Darl	Darp	Dars	Dxib	Daqr	Drpg	Dfri	Daql	Ddep	Dsax	Dxin	Dhop
K4.1	87.8	87.8	87.8	87.8	87.7	87.6	87.6	87.6	87.5	87.4	87.4	87.3	87.3	87.2	87.2	87.2	87.1	87.1	87.1	87.1	87.0	87.0	86.7
Dpim	88.1	87.7	87.4	87.9	88.9	88.2	87.6	88.7	88.2	86.3	87.3	86.0	86.4	88.9	87.3	87.8	86.5	85.8	86.7	87.5	86.0	88.0	88.1
Dper	89.2	89.0	89.5	87.7	88.5	88.4	89.4	86.9	89.1	88.6	89.8	88.0	87.9	88.7	89.7	89.1	87.9	87.9	89.1	88.2	88.2	89.2	89.7
Dmar	90.9	88.1	89.1	88.9	90.4	87.2	89.9	87.8	89.7	88.9	89.6	88.8	88.9	88.2	89.6	88.9	88.4	87.9	88.1	88.6	88.1	88.1	89.3
Dcae	91.9	89.6	91.5	91.9	94.6	89.7	93.5	90.2	91.4	93.6	92.7	93.3	93.2	89.3	92.5	91.4	92.7	92.4	93.3	97.0	93.4	92.6	92.8
Dhoh	91.2	87.6	91.2	91.1	96.0	90.0	93.1	89.1	90.9	91.9	92.6	92.9	93.9	88.9	92.5	90.5	91.9	91.9	93.3	94.0	91.8	90.9	93.4
Dgeo	88.7	87.4	89.8	90.2	89.0	93.4	90.0	91.7	88.2	88.7	89.9	88.9	90.0	92.9	89.7	88.2	88.7	89.0	89.1	88.9	88.4	88.9	89.3
Dros	86.6	98.0	87.8	86.8	88.1	85.9	87.3	85.4	86.2	87.0	86.9	87.3	87.1	85.9	86.9	87.5	86.3	85.5	87.7	89.5	86.5	86.8	87.5
Dpis	89.7	87.6	95.6	89.1	92.1	89.0	90.9	87.8	90.4	90.1	92.4	89.5	90.5	87.9	92.2	90.0	90.2	89.9	89.7	90.2	90.5	90.2	90.7
Dpro	90.1	88.0	95.1	89.0	91.6	89.2	91.1	87.9	90.6	90.3	92.4	89.5	90.0	88.3	92.3	90.1	91.0	90.2	90.1	90.6	90.7	90.6	91.1
Dnav	91.4	87.4	90.5	91.4	97.0	88.9	93.1	88.4	91.3	91.8	92.4	92.9	93.1	88.1	92.4	90.6	91.8	91.8	94.0	93.2	91.6	91.6	93.3
Dgra	91.4	87.5	91.1	91.9	94.9	89.4	93.6	89.7	90.9	92.7	93.0	92.9	93.4	88.9	92.9	90.2	93.5	93.0	93.3	94.9	92.7	91.4	93.1
Dfic	91.8	87.5	90.1	92.2	93.8	88.5	93.3	90.0	91.5	91.4	93.3	92.7	92.6	88.2	93.2	90.9	91.9	90.6	94.3	91.8	90.4	93.1	92.3
Dgob	92.0	87.0	91.2	91.7	93.4	89.4	93.0	89.5	92.0	91.5	92.3	91.7	92.7	89.9	92.1	90.8	91.6	91.5	92.2	92.8	92.0	92.2	92.2
Dmis	87.2	98.3	87.5	86.5	88.3	85.7	87.7	84.8	86.7	86.2	87.5	87.0	86.6	85.7	87.5	87.4	85.9	85.1	88.3	89.5	86.0	86.5	87.4
Daqc	92.7	89.5	91.6	92.3	94.9	89.5	93.7	90.2	92.2	93.2	92.5	93.3	93.0	89.5	92.3	91.8	92.8	92.3	93.3	96.6	93.1	93.0	93.2
Dmur	90.6	87.2	90.4	90.6	91.0	91.8	91.0	89.4	89.7	91.8	90.7	92.1	92.2	91.1	90.6	89.9	91.8	90.8	91.1	90.9	91.3	90.1	92.3
Dson	92.2	87.6	89.4	91.3	91.1	89.0	90.9	89.4	91.9	89.9	90.3	89.7	90.1	88.8	90.2	90.8	90.4	88.8	90.5	90.5	89.5	89.3	91.1
Dpap	87.7	86.2	87.2	87.7	88.2	87.9	87.3	87.8	87.6	86.2	86.5	85.6	86.1	88.1	86.4	87.3	86.8	85.8	85.8	87.1	86.1	87.2	87.4
Dind	91.9	88.6	91.9	92.0	92.9	89.5	96.3	89.9	91.0	93.0	94.9	91.7	92.7	89.6	94.7	90.7	92.1	91.2	93.1	95.6	92.1	92.5	93.5
Dalt	96.9	87.3	90.9	89.0	91.9	88.4	90.8	87.6	97.5	90.2	90.8	91.1	89.8	87.4	90.7	96.7	90.5	89.6	91.3	90.9	90.1	90.5	91.8
Dyav	88.1	86.5	86.9	87.5	87.9	87.7	87.6	87.9	87.6	87.2	86.3	86.9	87.0	88.6	86.2	87.3	87.1	86.5	86.4	87.4	86.9	87.5	87.2
Dalp	88.8	86.6	88.0	87.8	89.2	89.0	89.2	87.4	88.5	88.3	88.9	88.2	89.9	88.5	88.8	88.4	88.2	88.6	88.2	88.7	89.1	89.5	89.9
Drml		86.9	90.8	90.2	92.4	88.5	91.7	88.4	97.5	90.6	90.8	91.4	90.9	88.7	90.8	95.8	90.6	90.1	91.8	91.3	90.8	91.2	92.2
Dcel	87.0		87.7	86.6	87.8	85.9	87.2	85.1	86.7	86.4	87.1	86.9	86.5	85.8	87.1	87.5	85.6	85.0	88.0	89.7	85.9	86.8	87.1
Drph	90.8	87.6		89.4	91.0	88.7	91.4	87.9	90.4	89.5	91.9	90.2	91.0	88.2	91.8	90.4	90.2	89.9	89.1	90.2	90.1	90.7	92.2
Dyun	90.2	86.4	89.4		92.1	91.3	91.1	91.0	90.2	90.8	91.0	91.0	92.9	90.6	90.9	89.2	91.0	90.3	91.5	90.4	90.2	90.5	91.8
Ddes	92.5	87.7	91.3	92.3		89.0	92.6	88.6	92.3	92.6	92.6	93.7	93.6	88.5	92.4	91.5	92.6	92.0	93.7	93.2	93.0	92.0	93.7
Dapa	88.5	85.8	88.7	91.3	88.7		88.6	93.2	87.9	89.9	89.0	89.7	90.8	95.7	88.9	87.6	90.5	89.4	88.9	88.3	89.7	89.0	90.6
Drdr	91.7	87.1	91.4	91.0	92.4	88.6		88.9	90.8	91.3	97.3	90.0	91.9	88.0	97.3	90.3	91.0	90.1	93.8	93.2	90.0	91.7	92.4
Daet	88.4	85.0	87.9	91.0	88.1	93.2	88.9		88.1	89.3	89.0	88.7	89.1	93.1	88.7	87.7	89.1	88.6	88.6	88.0	88.6	89.4	89.6
Dcla	97.5	86.5	90.4	90.2	92.2	87.9	90.8	88.2		90.6	90.4	90.3	89.4	88.1	90.3	96.6	90.5	89.7	91.8	90.4	89.8	90.1	91.5
Dmrm	90.6	86.2	89.5	90.8	92.4	89.9	91.3	89.3	90.6		90.4	96.8	94.3	88.9	90.2	89.3	97.8	96.6	92.0	91.3	98.2	91.1	92.6
Dwul	90.8	87.0	91.9	91.0	92.3	89.0	97.3	89.0	90.4	90.4		89.8	91.3	88.3	100	90.0	90.6	89.8	93.2	92.1	89.4	91.7	92.5
Darl	91.4	86.8	90.2	90.9	93.6	89.7	90.0	88.7	90.3	96.8	89.8		95.0	88.5	89.5	89.9	96.3	95.0	91.4	92.3	96.4	90.9	93.0
Darp	90.9	86.3	91.0	92.9	93.4	90.8	91.9	89.1	89.4	94.3	91.3	95.0		90.2	91.1	89.4	93.7	94.1	91.8	92.5	94.5	91.1	93.4
Dars	88.7	85.7	88.2	90.6	88.1	95.7	88.0	93.1	88.1	88.9	88.3	88.5	90.2		88.0	87.4	89.1	89.0	87.9	88.1	89.0	89.0	90.6
Dxib	90.8	87.0	91.8	90.9	92.1	88.9	97.3	88.7	90.3	90.2	100	89.5	91.1	88.0		90.1	90.5	89.6	93.2	92.5	89.2	91.7	92.5
Daqr	95.8	87.4	90.4	89.2	91.4	87.6	90.3	87.7	96.6	89.3	89.9	89.9	89.4	87.4	90.0		89.1	88.2	90.5	91.8	88.3	90.8	91.0
Drpg	90.6	85.4	90.2	91.0	92.4	90.5	91.0	89.1	90.5	97.8	90.6	96.3	93.7	89.1	90.5	89.1		96.4	91.9	91.0	97.2	90.7	92.7
Dfri	90.2	84.9	89.9	90.3	91.7	89.4	90.1	88.6	89.7	96.6	89.8	95.0	94.1	89.0	89.6	88.2	96.4		91.2	89.6	97.7	91.1	92.0
Daql	91.8	88.0	89.1	91.5	93.7	88.9	93.8	88.6	91.8	92.0	93.2	91.4	91.8	87.9	93.2	90.5	91.9	91.2		94.0	91.3	91.1	91.8
Ddep	91.3	89.6	90.2	90.4	93.9	88.3	93.2	88.0	90.4	91.3	92.1	92.3	92.5	88.1	92.5	91.8	91.0	89.6	94.0		90.7	92.5	91.6
Dsax	90.8	85.7	90.1	90.2	92.8	89.7	90.0	88.6	89.8	98.2	89.4	96.4	94.5	89.0	89.2	88.3	97.2	97.7	91.3	90.7		91.6	92.5
Dxin	91.2	86.7	90.7	90.5	92.0	89.0	91.7	89.4	90.3	91.1	91.7	90.9	91.1	89.0	91.7	90.8	90.7	91.0	91.1	92.5	91.6		93.2
Dhop	92.2	87.0	92.2	91.8	93.6	90.6	92.4	89.6	91.5	92.6	92.5	93.0	93.4	90.6	92.5	91.0	92.7	91.9	91.8	91.6	92.5	93.2	

Dfic, *D.ficus*; Dfri, *D.frigens*; Dgeo*, D. geothermalis;* Dgob, *D.gobiensis*; Dgra, *D.grandis*; Dhoh, *D.hohokamensis*; Dhop, *D.hopiensis*; Dind, *D.indicus*; Dmrm, *D.marmoris*; Dmis, *D.misasensis*; Dmur, *D.murrayi*; Dnav, *D.navajoensis*; Dpap, *D. papagonensis;* Dper, *D. peraridilitoris;* Dpim, *D.pimensis*; Dpro, *D. proteolyticus;* Dros, *D.roseus*; D rdr, *D.radiodurans*; Drml, *D.radiomollis*; Drph, *D. radiophilus;* Drpg, *D. radiopugnans;* Dsax, *D.saxicola*; Dson, *D. sonorensis;* Dwul, *D.wulumuqiensis*; Dxin, *D. xingjiangensis;* Dxib, *D.xibeiensis* Dyav, *D.yavapaiensis*; Dyun*, D. yunweiensis;*

4.4 Transfer of *Bacillus cereus* spores from packaging papers to food

Bacteria of the *Bacillus cereus* group are the only pathogens that are regularly found in paper and thus they are of special interest when assessing the risk caused by packaging paper to food safety. Therefore, we wanted to quantify the transfer of *B. cereus* spores from packaging paper to food. To do this, a green fluorescent protein (GFP) labelled derivative of *Bacillus thuringiensis* (Bt 407Cry$^-$ [pHT315Ω(papha3-gfp)], abbreviated BT-1, Figure 8) was constructed and used to prepare paper containing spores of this strain. The paper sheets were made employing a technique allowing fibre formation similar to those of industrially manufactured food packaging papers. Chocolate and rice were the recipient foods when transfer of the labelled spores from the packaging paper to food was measured.

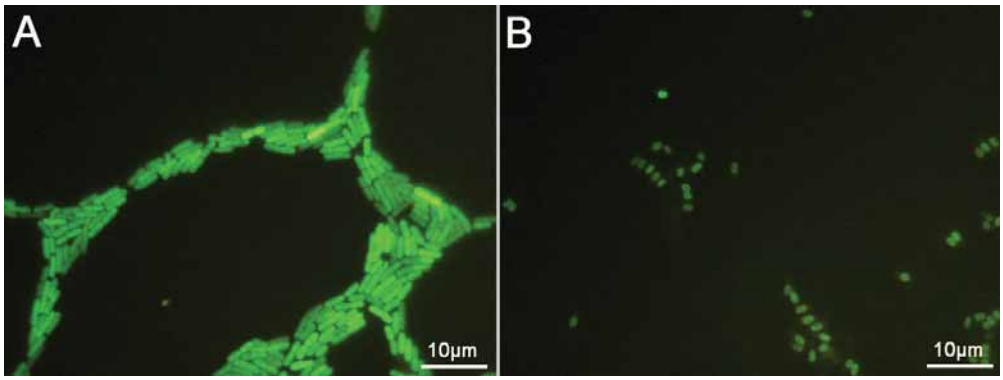


Figure 8. Fluorescence microscopic images of vegetative cells (A) and spores (B) of the *B. thuringiensis* strain BT-1 (Bt 407Cry$^-$ [pHT315Ω(papha3-gfp)]).

4.4.1 Only surface exposed *B. cereus* spores were transferred from paper into food

Dry rice was exposed to the paper containing spores of BT-1 for 10 d at 40°C. These are the conditions recommended by European Commission for safety assessment of food contact materials (Anonymous, 1997). Because chocolate melts at 40°C, the exposure to the BT-1 containing paper was executed at 20°C. The lower exposure temperature was compensated by extending the contact time to 30 days. The transfer was studied at ambient air humidity, which was 10% at 40°C (rice) and 30% at 20°C (chocolate). To test the effect of high moisture on the transfer, the exposures were repeated at the air humidity of 60%. The contact areas for rice and chocolate were 4.0 and 0.95 cm^2/g of the food, respectively. These are in the same range with real food packages (Table 2 of Paper II).

The vast majority of the spores remained immobilized in the packaging paper, only 0.001 - 0.03% transferred to the foods (Table 3 of Paper II). As we noted in the Paper II, this amount

of transferred spores was small compared to the amounts commonly found in foods. Thus, it is likely that the spores transferring from paper to food cause no risk for food safety.

We noticed that 0.03 - 0.1% of BT-1 spores in the paper transferred to fresh agar surface within five minutes of contact (Tables 3 and 4 of Paper II). Earlier, Johansson *et al.* (2001) observed similar amount of transfer using 20 h contact time. Our experiments showed that the transfer from paper to agar surface in five minutes was more than to what transferred food during 10 - 30 days of exposure. This means that transfer of spores from packaging paper to food is restricted to those exposed on paper surface and detectable with contact agar method. This result is in line with the results of Suominen *et al.* (1997). They found that bacteria inside the cellulosic fibre web were unable to grow or to move, not even during extended contact with moist food. Thus, the relatively simple contact agar method can be used for estimating the amount of potentially transferring spores. My results indicate that the transfer to food would most likely be less than observed in the contact agar experiment.

4.5 Biological function of cereulide, the emetic toxin of *Bacillus cereus*

4.5.1 Cereulide affects the K^+ homeostasis of *B. cereus* cells.

I wanted to know how cereulide operates in *B. cereus* cells and if it acts differently on the cells of cereulide producing and non-producing strains. Figure 9 shows the leakage of K^+ ions on-line measured from the cells of two *B. cereus* strains suspended in K^+ free medium. When cereulide was added into the cell suspension of strain ATCC 14579^T (cereulide non-producer, Figure 9, A), a rapid increase in the extracellular concentration of K^+ was seen. This shows that K^+ ions leaked out from this bacterium upon exposure to cereulide. But when an energy source (glucose) was added, the extracellular K^+ concentration declined back to the original level within three minutes. This shows that when energy was available, *B. cereus* ATCC 14579^T cells pulled the lost K^+ ions back into their cells. When a cereulide producing strain F4810/72 (Figure 9, B) was similarly tested, a different response was seen. Some K^+ ions was leaking from the cells, but exposure to 20 nM cereulide induced no additional leakage of K^+ ions. The addition of glucose resulted in to uptake of K^+ ions also by the cells of strain F4810/72, but less prominently than with the strain ATCC 14579^T.

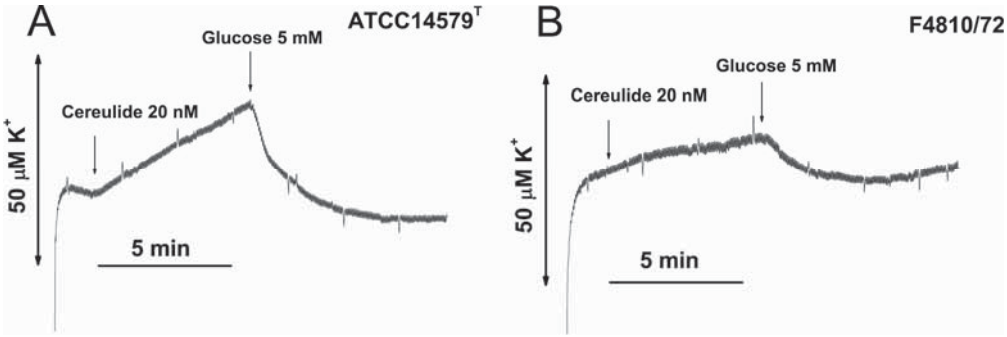


Figure 9. On-line measurement of cereulide induced efflux of K^+ from *Bacillus cereus* cells.
Actively growing, washed cells of the strain ATCC 14579^T (Panel A) and F4810/72 (Panel B) were suspended in a buffer with no added K^+. The cell suspension (0.2 mg dry weight) was in a cuvette fitted with a K^+ selective electrode for on-line monitoring of the extracellular concentration of K^+ ions. Where indicated, 20 nM cereulide and 5 mM glucose were added.

I further investigated potassium trafficking induced by cereulide in two cereulide producing (F4810/72 and NS58) and two non-producing (ATCC 14579^T and B319) strains of *B. cereus*, and also in *B. subtilis* DSM 347 and *Escherichia coli* ATCC 51739 (Table 1 of Paper IV). Responses of the cereulide producing strains of *B. cereus* towards added cereulide differed markedly from those of the other strains of *B. cereus* or from those of *B. subtilis* DSM 347. Low concentration of added cereulide (20 nM) resulted into a strong K^+ efflux, corresponding to a loss of 50 % – 80 % of the cellular stores of K^+, from the cereulide non-producing *B. cereus* strains and from *B. subtilis* DSM347 within 180 min. In contrast, only minor K^+ leakage, < 10 % of the cellular stores of K^+, occurred from the cereulide producing *B. cereus* strains F4810/72 and NS58, and only after an exposure to a 10× higher concentration of cereulide, 200 nM. Exposure to cereulide induced no measurable K^+ efflux from *E. coli* ATCC 51739, possibly because the outer membrane of Gram-negative bacteria excluded the entry of the hydrophobic molecule cereulide. These results show that cereulide producing *B. cereus* strains possess means for preventing the cereulide mediated leakage of potassium like that occurring in cereulide non-producing *B. cereus* and in *B. subtilis*.

The effect of cereulide on the membrane potential of *B. cereus* cells was measured using the fluorogenic membrane potential indicator dye JC-1 (Figure 10). Exponentially growing culture of *B. cereus* NS58, a cereulide producer, was harvested, washed with and resuspended in Na-phosphate buffer (1 mM, pH 7.3). The buffer had no added K^+, but the residual K^+ concentration from the growth medium was likely 0.1 – 0.2 mM, alike measured

in Figure 1 of Paper IV for similarly washed cells of *B. cereus*. Staining with JC-1 differentiates cells with high membrane potential (140 to 160 mV, negative inside) by their yellow fluorescence from cells with low membrane potential (<100 mV), fluorescing green (Reers *et al.* 1995). Most cells in Figure 10, Panel A fluoresced green, with some brightly yellow cells with high membrane potential. After cereulide was added (Panel B), all cells responded by fluorescing yellow, meaning that their membrane potential increased. This indicates cereulide mediated efflux of K^+ ions from the cytoplasm, as shown in Figure 9 for the strain ATCC 14579^T, down the concentration gradient from the cells to the extracellular space. The efflux of positive ions leaves the interior of the cell more negative and thus increases the membrane potential.

When cells of *B. cereus* NS58 were suspended in a buffer with high K^+ concentration (120 mM, close to cytoplasmic concentration, Panel C), cells on average displayed a higher membrane potential than in the plain buffer with no added K^+. When a high concentration of cereulide was added together with K^+ ions (Panel D), all cells lost their transmembrane electric potentials, indicating that cereulide converted the cell membrane permeable for K^+, resulting into electrochemically driven flow of the positively charged K^+ ions into the cells. This flow of positive ions decreased the net negative charge inside the cells. Similar decrease of membrane potential after addition of cereulide into a high K^+ medium was also shown with isolated mitochondria (Teplova *et al.* 2006), the eukaryotic cell organelles that resemble bacteria. Tempelaars *et al*. (2011) showed that 9 µM cereulide caused depolarization of Gram-positive bacteria also in somewhat lower concentration of K^+ (50 mM K^+, pH 8.5).

Next, I studied the effect of energy status of the *B. cereus* cells on their membrane potential. In presence of glucose (Figure 10, Panel E, background concentration of K^+ ~0.2 mM), all *B. cereus* cells became bright yellow fluorescing. The high membrane potential shows that *B. cereus* cells had an energy source to create a proton gradient. When cereulide was added together with glucose, cells fluoresced green showing that the membrane potential generated by glucose was consumed. Thus, 70 µM added cereulide neutralized the membrane potential of *B. cereus* cells apparently similarly whether external K^+ concentration was 120 mM (Panel D) or 0.2 mM (Panel F). The fact that the cell lost membrane potential in the presence of cereulide indicates that high amount of K^+ must have entered the cells and thus cereulide can mediate K^+ influx from an extremely low extracellular concentration. Similarly to this, Teplova *et al*. (2006) measured that cereulide caused influx of K^+ ions into isolated, energized rat liver mitochondria from low concentration (~3 mM) and that cereulide

mediated swelling of mitochondria (indicating influx of K^+) in isotonic media with K^+ concentrations as low as 0.2 mM.

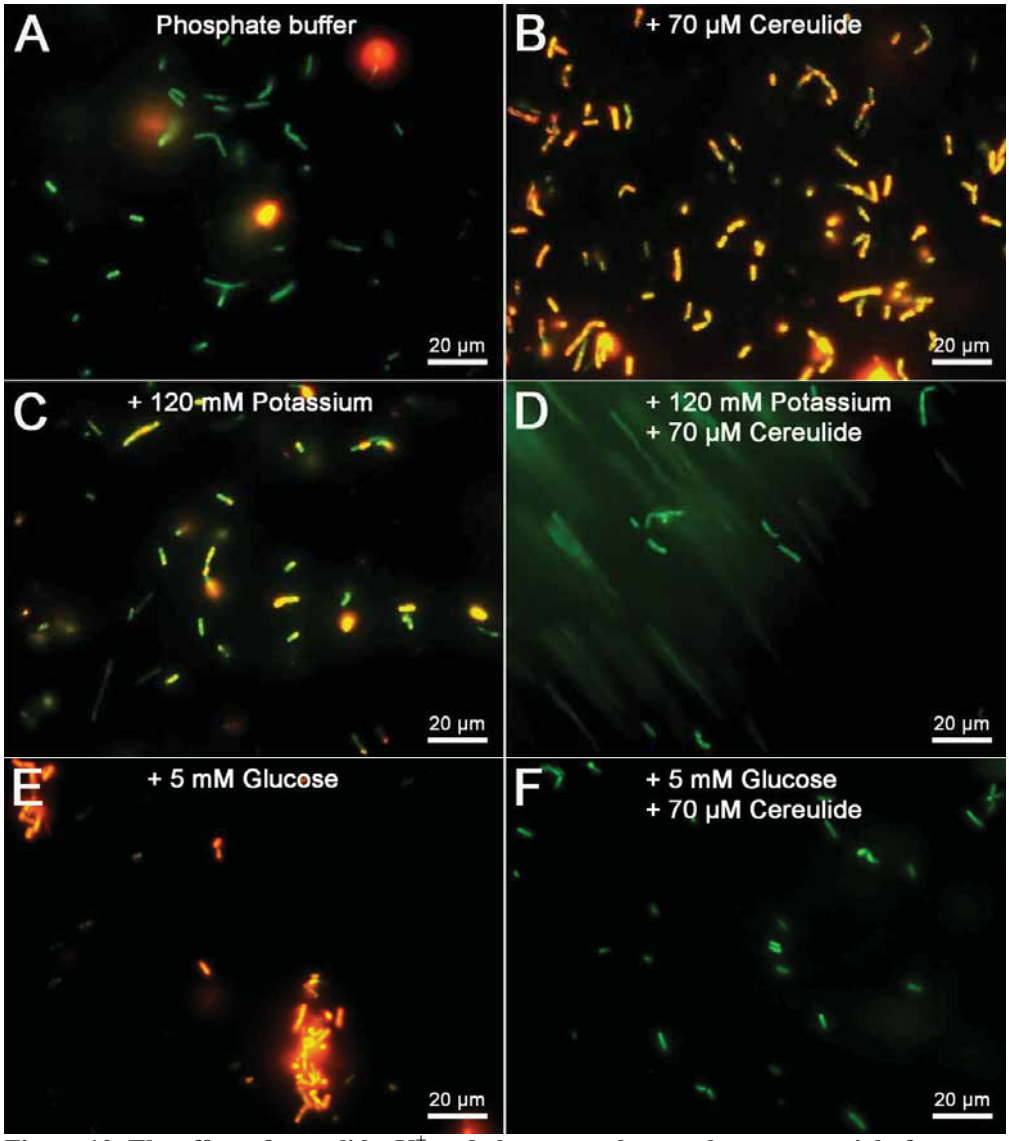


Figure 10. The effect of cereulide, K^+ and glucose on the membrane potential of cereulide producing *Bacillus cereus* NS58.
Cells from exponential growth phase were suspended in 1 mM Na-phosphate buffer (No K^+ ions or energy source, pH 7.3). Additions indicated in the figure were made, membrane potential sensitive fluorogenic dye JC-1 was added and the result was observed with epifluorescence microscope. Green color indicates low (< 100 mV) and yellow high (140 – 160 mV) membrane potential.

The membrane potential changes, indicated by changes of fluorescence output of JC-1 (Figure 10), reflect the changes of transmembrane electric potentials, whereas the electrode measures K^+ ion concentration (Figure 9). Although 20 nM cereulide caused no measurable K^+ efflux from strain F4810/72 or from strain NS58 (Figure 9 and Paper IV, Table 2), high concentration of cereulide (3500×dose, 70 µM, Figure 10, Panel B) seemed to do so. It would be very interesting to test cereulide non-producing *B. cereus* strains to see if their membrane potential changes in response to smaller cereulide concentrations than strain NS58. Tempelaars *et al*. (2011) presented results supporting this hypothesis: 9 µM cereulide depolarized the cells of cereulide non-producing *B. cereus* ATCC10987 completely, but the cells of cereulide producing strain F4810/72 only partially.

4.5.2 Cereulide helps its producer to compete in potassium deficient environment

To find out if cereulide producing strains gain any competitive advantage over non-producers in low K^+ environments, an GFP labeled indicator strain BT-1 (*B. cereus sensu lato*), was cocultured with cereulide producing and with non-producing *B. cereus* strains. Two growth media were used, one rich and one deficient for K^+. Figure 11 shows that the cereulide producing strains F5881, NS58 and F4810/72 suppressed the growth of BT-1 almost totally (90 to 100%) under K^+ deficient conditions, *i.e.* in TSB-Na medium (TSB where K-phosphate was replaced with Na-phosphate, residual K^+ concentration being ~1 mM) but not in full strength TSB (contains ~30 mM K^+). Cereulide non-producing ATCC 14579^T also mildly suppressed the growth of BT-1 in co-culture, but did so similarly in TSB and in TSB-Na. The results show that the cereulide producing strains were more efficient competitors than the cereulide non-producing ATCC 14579^T in a medium where K^+ concentration was growth limiting.

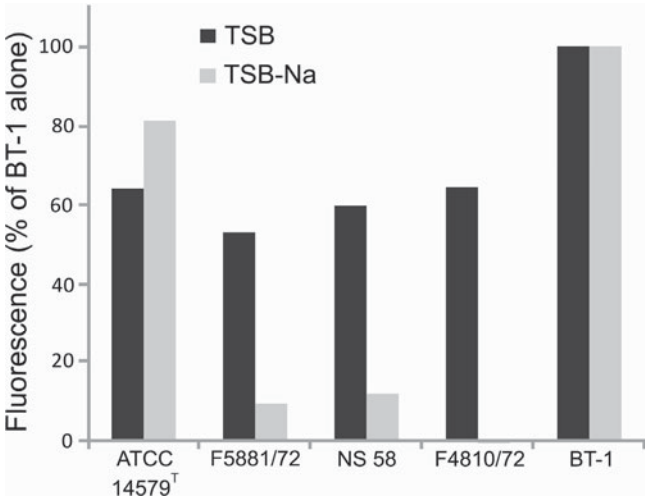


Figure 11. Propagation of the GFP labeled *B. thuringiensis* BT-1 in co-cultures with cereulide producing and non-producing strains of *B. cereus*. Actively growing *B. cereus* cultures were serially diluted in TSB or TSB-Na. Each of these cultures (agonists, 10^{-3} dilution) was mixed 1:1 (v/v) with similarly diluted culture of the strain BT-1. These mixed cultures were dispensed into the wells of microplates and after 2 d incubation (28°C, 120 rpm) fluorescence of the wells was read (ex 485 nm/em 535 nm). The figure shows a summary of the data presented in Table 2 of Paper iV.

To see if bacteria gain any advantage of added cereulide, I measured the growth rates of nine strains of *B. cereus* and three other species in the presence and absence of added cereulide in two media, TSB and TSB-Na (Table 3 of Paper IV). In TSB-Na (deficient for K^+), the maximal growth rate (μ_{max}) of each of the five cereulide producing strains of *B. cereus* (NS58, F4810/72, F5881/94, B318 and GR177) was higher (6% to 26%, $p = 0.02$) in the presence of added cereulide (2 μM) than when none was added. The μ_{max} of cereulide non-producing strains of *B. cereus* (ATCC 14579^T, B319, B117 and BT-1) or of other tested strains (*B. subtilis* DSM 347, *E. coli* ATCC 51739 and *P. putida* C3024) neither increased nor decreased from the added cereulide in either of these two media. Thus, cereulide was advantageous for cereulide producers in low potassium environment and was not growth inhibiting towards cereulide non-producing *B. cereus* or other species under these conditions (TSB or TSB-Na).

Cereulide thus seems to have a function in K^+ uptake: cereulide molecule binds K^+ at one side of membrane and eletcrophoretically transports it to the other side. The direction of the K^+ flow will be determined by the electrochemical gradient across the membrane and the equilibrium potential can be calculated with Nernst equation ($\Delta\Psi = 59 \times \log [K_o]/[K_i]$) when the ion concentrations are known. So, if the cells are in good nutritional shape and have high membrane potentials ($\Delta\Psi$ = -140 to -160 mV, as shown in Figure 10, panel E), cereulide can transport K^+ ions into the cells against a 250 - 500× concentration gradient, *i.e.* from an

extracellular concentration ($[K_o]$) of 0.4 – 0.2 mM to the intracellular concentration ($[K_i]$) of 100 mM. Extracellular concentrations of ≤ 0.2 mM are common in natural environments.

Cereulide mediated potassium transport across biological membranes is a well documented process with mitochondria. Accumulation of potassium into mitochondria is seen as swelling of these organelles (Sakurai *et al.* 1994, Mikkola *et al.* 1999). In those studies mitochondria were observed inside living cells with high cytoplasmic K^+ concentration (~140 mM). Teplova *et al*. (2006) showed the transport with isolated mitochondria exposed to low K^+ environment. They also noticed that valinomycin, a well known K^+ ionophore produced by certain strains of *Streptomyces,* acted similarly but with an lower efficiency than that of cereulide. Similar K^+ transporting mechanism has also been shown for enniatins and beauvericin, potassium binding mycotoxins produced by *Fusarium* sp. (Tonshin *et al.* 2010).

Shaheen *et al*. (2006) measured amounts of cereulide produced in infant foods. They reconstituted commercial infant food formula at different concentrations with drinking water and measured the production of cereulide by *B. cereus* strain F4810/72 during 24 h incubation. They reported that cereulide production was almost inversely correlated with the dry matter content of the food, *i.e.* almost 50 times more cereulide accumulated in diluted formula containing only 10 g per l dry matter compared to the formula containing 150 g per l, as instructed by the manufacturer. Our findings seem to explain these findings: In diluted infant food the K^+ concentration was only 1/15 (~4 mM) of the amount in undiluted infant food formula (~60 mM, www.fineli.fi, accessed 16.12.2010). If *B. cereus* produces cereulide in order to efficiently utilize K^+ from the environment, it needs more cereulide in the diluted than in the standard infant food. My results indicate that the effect of K^+ concentration on the cereulide production should be studied systematically.

4.5.3 Cereulide enhances biofilm formation of *Bacillus cereus*

As shown in Figure 12, biofilm formation of *B. cereus* is strongly strain dependent. Bulky biofilms were formed by two cereulide producing strains, F5881/94 and NS58. Instead, the *B. cereus* type strain ATCC 14579^T produced no biofilm under these conditions (TSB, 28°C, 120 rpm). Similar to this, Wijman *et al*. (2007) noted that the strain ATCC 14579^T produced no biofilm in rich LB-medium. In addition, these authors reported that *B. cereus* formed biofilm at the air-liquid interphase, like also shown in Figure 12.

Auger *et al*. (2009) reported that emetic *B. cereus* strains produce no biofilm on PVC in LB-medium. My findings (paper IV and this thesis) show that emetic strains F4810/72, NS58,

F5881/94 and B318 were able to grow as biofilms under several conditions (TSB, diluted TSB, Sporulation medium) and on many different surfaces (polystyrene, glass, as free-floating pellicles).

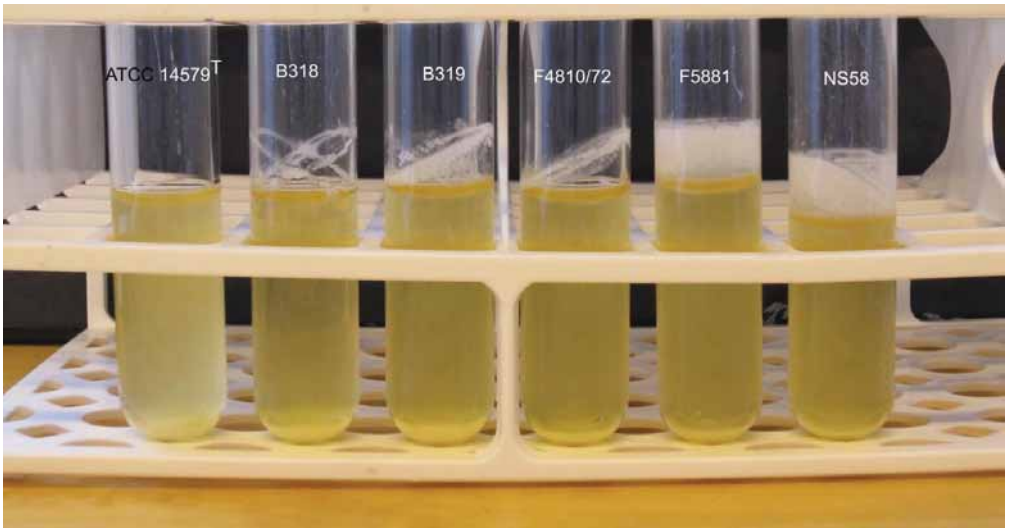


Figure 12. Biofilm formation by strains of *B. cereus*.
The strains were grown in glass tubes for 2 d in TSB medium at 28°C (shaking 120 rpm).

To explore the impacts of cereulide on biofilm formation by *B. cereus*, I added cereulide into cultures of cereulide producing and of non-producing *B. cereus* strains. Figure 13 and Figure 3 of Paper IV show the results of pellicle formation assays. Added cereulide induced biofilm formation both in TSB (strains F4810/72 and NS58, Figure 13) and in the Sporulation medium (strains ATCC 14579^T, B318, NS58, F4810/72 and F5881, Figure 3 of Paper IV). The biofilm promoting effect was stronger on cereulide producing strains than with the type strain *B. cereus* ATCC 14579^T, especially in TSB medium. Added K^+ (150 mM) prevented biofilm inducing effect of cereulide on the type strain but not on the cereulide producing strains (Figure 3 of Paper IV). It is possible that weaker pellicle formation of ATCC 14579^T in TSB than in Sporulation medium was due to the difference K^+ concentration (~30 mM in TSB vs. ~15 mM in Sporulation medium).

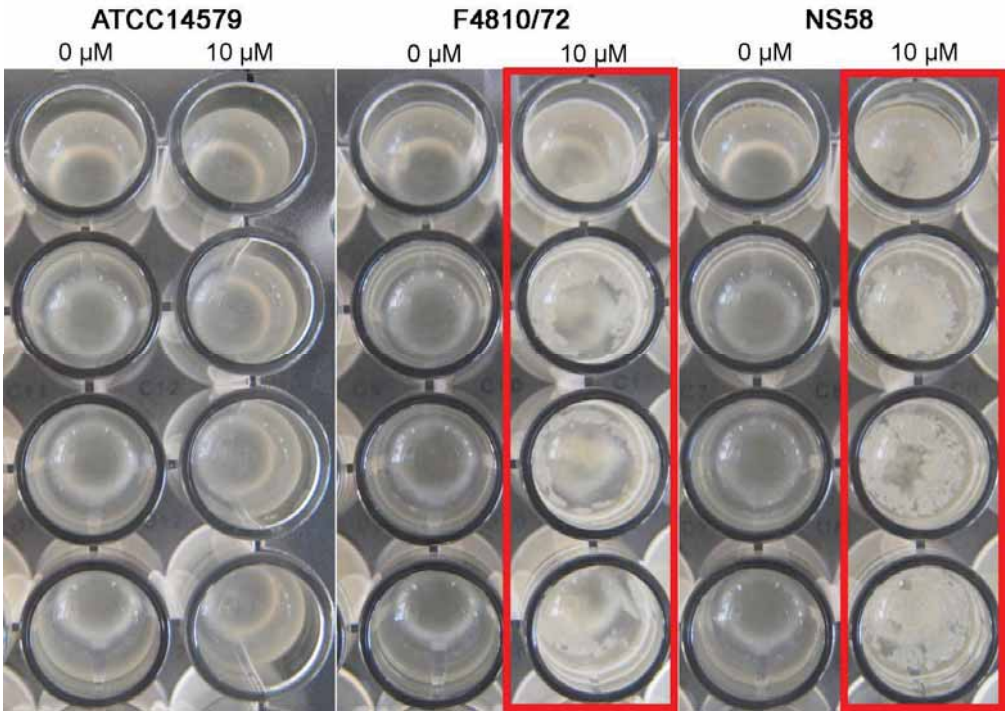


Figure 13. Cereulide induces pellicle formation of *Bacillus cereus*.
Actively growing *B. cereus* cultures in TSB were diluted 1:10 with sterile TSB. The obtained suspensions were dispensed into the wells of a microplate and 0 or 10 µM cereulide was added. After incubation of 17 h (28°C, 120 rpm) the plate was photographed. Wells with pellicles are highlighted with red rectangles.

Figure 14 shows phase contrast microscopic images of preparations from pellicle formation assays similar to those presented in Figure 13 and in Figure 4 of Paper IV. In diluted TSB almost all cells in the cereulide treated wells of the cereulide producing strains (B318, F4810/72, F5881/94 and NS58) formed massive aggregates, whereas in wells with no added cereulide mostly single cells or small clumps were seen. Cereulide non-producers, ATCC 14579^{T} and B319, did not respond to cereulide addition as strongly as the cereulide producing strains: In cereulide treated wells, the cells were more attached to each other than in wells without cereulide, but the cell clumps were much smaller than those seen with the cereulide producing strains. Figure 15 shows the ultrastructure (FESEM images) of pellicles formed by *B. cereus* strains F4810/72 (panels A and B) and F5881/94 (panels C and D). Dried EPS is visible between the *B. cereus* cells (panels B and D).

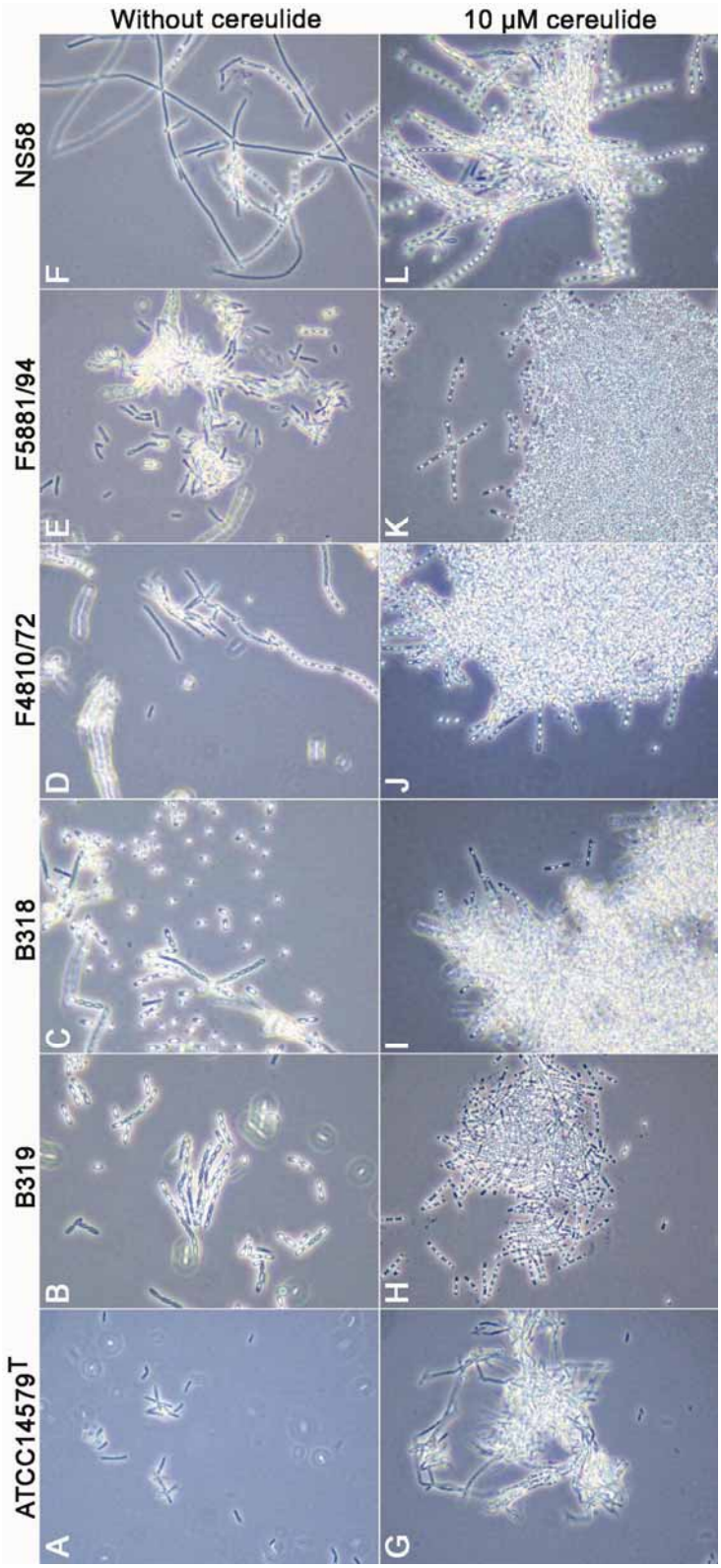


Figure 14. Phase contrast microscopic images of *B. cereus* cultures with and without added cereulide.
Overnight cultures of *B. cereus* in TSB were diluted ten fold with sterile drinking water and 0 or 10 µM of cereulide was added. After incubating 23 h (28°C, 120 rpm) the cultures were inspected with a phase-contrast microscope. The strains ATCC 14579^{T} and B319 produce no cereulide, the strains B318, F4810/72, F5881/94 and NS58 are cereulide producers.

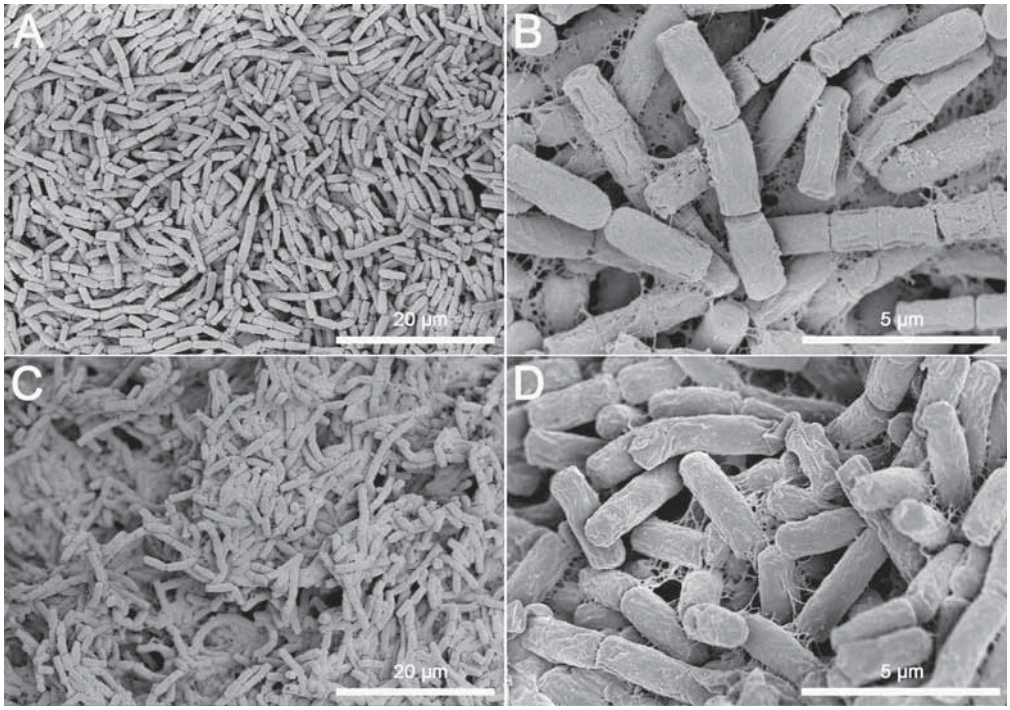


Figure 15. Field emission scanning electron micrographs (FESEM) of pellicles of *B. cereus* strains F4810/72 (A and B) and F5881/94 (C and D) (Courtesy of Mari Raulio). The pellicles were grown as described in Paper IV. The samples were fixed and the images taken as described by Raulio *et al.* (2006).

The effect of cereulide on biofilm formation of *B. cereus* was measured quantitatively using the crystal violet assay (Figure 16, Figure 17 and Figure 2 of Paper IV). The biofilm accumulation by the two test strains, NS58 and F5881/94, correlated with the amount of cereulide added (Figure 16). In Figure 2 of Paper IV, five *B. cereus* strains and two culture media were used. Biofilm yield by all test strains (ATCC 14579^T, B319, F4810/72, NS58 and F5881/94) increased in dilud TSB when cereulide was added. In full strength TSB, the same effect was seen with all strains expecting ATCC 14579^T. This phenomenon also occurred with washed cells in phosphate buffer. The results (Figure 17) show that the accumulated biofilm originated from pre-existing cells that attached to walls of the wells after cereulide was added. Cereulide thus enhanced attachment of *B. cereus* cells similarly independent on the availability of carbon substrate (0 or 10 mM glucose) or the concentration of K^+ (0 or 60 mM) of the medium.

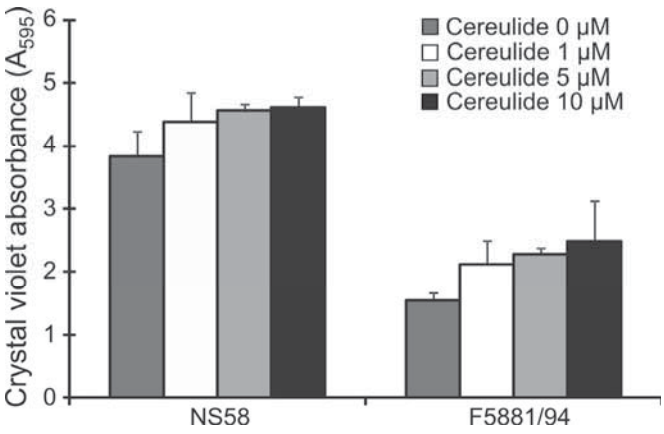


Figure 16. Biofilm yield by *B. cereus* correlates the amount of added cereulide. Actively growing *B. cereus* cultures in TSB were dispensed into wells of a polystyrene microplate and indicated amounts of cereulide were added. After 24 h incubation (28°C, 120 rpm) the wells were emptied and the biomass adhered to the walls of the wells was stained with crystal violet.

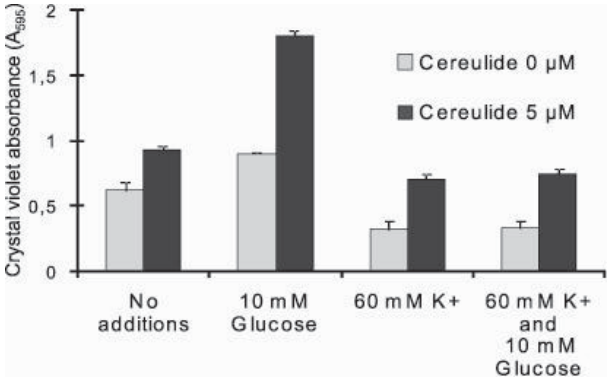


Figure 17. Cereulide increases surface attachment by *B. cereus*. Actively growing cells of the cereulide producing strain F5881/94 were washed, suspended in 1 mM Na-phosphate buffer (pH 7.3) to density of A_{600}=0.8 and dispensed into the wells of a microplate. Indicated amounts of glucose, K^+ ions and cereulide were added. After 18 h incubation (28°C, 120 rpm) the biomass adhering the walls of the wells was stained with crystal violet.

It was shown that compounds causing K^+ leakage, including surfactin produced by the organism itself, induced biofilm formation of *Bacillus subtilis* strain NCIB 3610 (Lopez *et al.* 2009). Similarly to our results with *B. cereus* ATCC 14579^T, Lopez *et al.* (2009) showed that high concentration of K^+ (150 mM) prevented pellicle formation by *B. subtilis* NCIB 3610. In contrast, high concentration of K^+ did not prevent pellicle formation by cereulide producing *B. cereus* strais (Figure 4 of Paper IV). Summing up, as we discussed in Paper IV, my results indicate that the mechanism by which cereulide induces biofilm formation in cereulide producing *B. cereus* strains is different from that in *B. cereus* ATCC 14579^T and from that described by Lopez *et al*. (2009).

Taken together, the results presented in this thesis and in the Paper IV clearly show that cereulide enhances the attachment of *B. cereus* cells to each other and onto abiotic surfaces. Cereulide induced pellicle formation was seen in three media: TSB (Figure 13), diluted TSB

(Figure 14) and Sporulation medium (Figure 4 of Paper IV) using five *B. cereus* strains. The increase in surface growth was documented in three media: TSB (Figure 16, Figure 2 of Paper IV), 10× diluted TSB (Figure 2 of Paper IV) and phosphate buffer (Figure 17).

5. Conclusions

1. I showed that *Meiothermus* spp. are major colonizers in paper machines. Several other primary-biofilm forming isolates were also shown to belong to the phylum *Deinococcus/Thermus* (including a novel genus, *Deinobacterium*). It thus seems that members of this phylum are well adapted to warm, aqueous industrial environments. I predict that they are also able to colonize other man-made environments, such as power plants and pipe systems for warm water.

2. I showed a connection between end product defects and biofilms in the wet-end of paper machines and that the bacterial involvement in the formation of end product defects can be demonstrated by DNA extraction and qPCR analysis.

3. We developed a qPCR method for detection of *Pseudoxanthomonas taiwanensis*. I showed that this bacterium is a common contaminant in paper machines and it often makes up a high proportion of the bacteria in process waters.

4. I described a novel bacterial genus, *Deinobacterium*. The type species *Deinobacterium chartae* is extremely radiation resistant and capable of primary-biofilm production at paper machine conditions.

5. I isolated 48 strains, representing more than 15 genera, of primary-biofilm formers from paper machines. Eight of these genera were earlier known as colonizers of geothermal springs. This shows that bacteria adapted to thermophilic and oligotrophic environments are able colonize man-made habitats beyond large geographical distances.

6. I showed that only a minute proportion (0.001% - 0.03%) of *Bacillus cereus* spores transferred from food packaging papers into food.

7. I showed that the numbers of *B. cereus* spores transferring from packaging paper to food was very low compared to the numbers naturally occurring in foods. Spore transfer from packaging paper is unlikely to cause any risk for food safety.

8. I suggest that organisms producing cereulide utilize this substance for importing potassium from low potassium environment. Cereulide seems to enable utilization of

potassium over vast concentration range (over 500 fold from 0.1 to 120 mM). This is the first time when small carrier molecule is connected to transport of potassium.

9. I showed that cereulide producing *B. cereus* strains are more competitive than non-producers in potassium deficient environment. Thus the natural habitat for these organisms may be in potassium poor environments, such as the extracellular space inside living plants.

10. I showed that nanomolar concentrations of cereulide induce leakage of potassium from *B. cereus* not producing cereulide and also from *B. subtilis*, but not from cereulide producing *B. cereus* strains. Thus cereulide producing strains have some means to prevent potassium leakage caused by cereulide.

11. I showed that cereulide enhances biofilm formation of *B. cereus*. High extracellular potassium concentration did not prevent biofilm formation, which implicates that potassium leakage is not the signal triggering biofilm formation in cereulide producing *B. cereus*.

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Vantaa, 17.4.2011

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